



Generation and characterization of dapsone- and nitroso-dapsone-specific T-cells: an insight into the molecular pathomechanism of dapsone hypersensitivity syndrome.

This thesis is submitted in accordance with the requirements of the University of Liverpool
for the degree of Doctor of Philosophy by

Abdulaziz Alzahrani

January 2018

Declaration

I declare that the work presented in this thesis is all my own work and has not been submitted for any other degree

.....

Abdulaziz Alzahrani (B.Pharm, MSc Clinical pharmacology)

***Dedicated to
(My wife Nadiah, My daughters
(Aljori, Ragad, Lubna and Lama)***

First and foremost, I would like to thank Allah for awarding me patience with endurance, wellbeing, and knowledge to continue working for this PhD thesis. I would like to express my immense gratitude and thankfulness to my supervisors Professor Dean Naisbitt and Professor Kevin Park for giving me the chance to join their group, and for their supervision, encouragement, support and constructive guidance throughout my entire PhD journey. Prof. Dean Naisbitt's passion in science and patience to overcome obstacles inspired me as a developing scientist and as a human. Despite Prof. Naisbitt's busy schedule, he always gave me time whenever I needed, which I greatly appreciated.

Sincere and special thanks should go to Dr Monday Ogebe for his patience and guidance as well as his support and care, and for teaching me most of the basic cell culture techniques in such an easy and enjoyable way. I gratefully acknowledge Dr Xiaoli for her positive insight and suggestions in the mass spectrometry work. Thanks must go to my financial sponsors the Saudi Government and University of Liverpool who have enabled this work to be completed. A special acknowledgement should go to my colleagues in the AL-BAHA University who helped me to successfully finish my sponsorship paper work. Also, I convey my thanks to our clinical team, including Professor Munir Pirmohamed, Dr Ana Alferovic and the research nurses, and the volunteers who participated in this study. A lot of thanks to the Chinese members for permitting me to use their patients during my research.

Many thanks to all my friends in Prof. Naisbitt group who were always ready to help and provided me the most unique and enjoyable atmosphere during my time at the university. Not forgetting Paul, Arun, Khetam, James and Gole for their support in all aspects, especially listening to my presentation.

My grateful thanks go to Dr Andrew Gibson, Mr John Farrell, and Dr Lee Faulkner for their guidance, supporting which helped me to convey it to my colleagues in the lab. Last but not least, I express my deep wholehearted gratitude to my brothers and sisters, for their continuous support, care and prayers.

Finally, from all my heart I would like to express my great appreciation and thankfulness to my lovely wife Nadiah Alzahrani, who left her job and everything she loves behind to join me without any hesitation. Nadiah is always giving me an extraordinary support, love, care and encouragement during my whole PhD journey; without her support I would not have been able to reach this stage of my career. My daughters, Aljori, Ragad, Lubna and Lama thank you for your lovely smiles, fun and continuous love which helped me get through the difficult days.

Contents

<u>TITLE:</u>	I
<u>DECLARATION:</u>	II
<u>ACKNOWLEDGMENTS:</u>	III
<u>CONTENTS:</u>	IV
<u>ABBREVIATIONS:</u>	V
<u>PUBLICATIONS:</u>	IX
<u>ABSTRACT:</u>	X
<u>CHAPTER 1 GENERAL INTRODUCTION:</u>	1
<u>CHAPTER 2 SYNTHESIS NITROSO-DAPSONE AND PRIMING OF NAIVE T-CELLS TO DAPSONE AND NITROSO DAPSON:</u>	54
<u>CHAPTER 3 CHARACTERISATION OF DDS-AND DDS-NO-SPECIFIC T-CELL CLONES FROM HLA-B*13:01 NEGATIVE HEALTHY DONOR:</u>	87
<u>CHAPTER 4 GENERATION AND CHARACTERISATION OF DDS-AND DDS-NO-SPECIFIC T-CELL FROM 2 PATIENTS HLA-B*13:01 POSTIVE:</u>	109
<u>CHAPTER 5 DEFINING THE ANTIGENICITYAND IMMUNOGENICITY OF TEST COMPOUNDS USING A MOUSE MODEL:</u>	143
<u>CHAPTER 6 FINAL DISCUSSION:</u>	161
<u>BIBLIOGRAPHY:</u>	168

Abbreviations:

3DDS 3,3 -diaminodiphenyl sulfone

ACN Acetonitrile

ADR Adverse drug reaction

ALP Alkaline phosphatase

amu atomic mass unit

APC Antigen presenting cell

APC Antigen presenting cells

ANCAs Anti-neutrophil cytoplasmic IgG antibodies

CD Cluster of differentiation

CFSE Carboxyfluorescein diacetate succinimidyl ester

CPM counts per minute

CRM Chemically reactive metabolite

CSA Cyclosporin

Ct Cycle threshold

CTL Cytotoxic T lymphocyte

CYP Cytochrome P450 enzyme

DAMP Damage associated molecular pattern

DDS Dapsone, 4,4-diaminodiphenylsulfone

DHS Dapsone hypersensitivity syndrome

DC Denteric cell

DDE 4,4- diaminodiphenyl ether

DDS-NHOH dapsone-hydroxylamine

DDS-NO (Nitroso-dapsone)

DDT 4, 4 diaminodiphenyl sulfide

DDT 4-acetyl-4-aminodiphenyl thioether

DHR Drug hypersensitivity reaction

DILI Drug-induced liver injury

DMSO Dimethyl sulfoxide

DNA Dioxynucleic acid

DNCB Dinitrochlorobenzene

DRESS Drug reaction with eosinophilia and systemic symptoms

EBV Epstein-Barr virus

EDTA Ethylenediaminetetraacetic acid

ELISpot Enzyme-linked immunospot

ELISA Enzyme-linked immunosorbent assay

FA Formic acid

FACS Fluorescence activated cell sorting

FBS Foetal bovine serum

FITC Fluorescein isothiocyanate

GB granzyme B

GM-CSF Granulocyte-macrophage colony-stimulating factor

GSH Reduced glutathione

HBSS Hanks balanced salt solution

HEPES Hydroxyethyl piperazineethanesulfonic acid

HIV Human immunodeficiency virus

HLA Human leukocyte antigen

HPLC High-performance liquid chromatography

IFN- γ Interferon-gamma

IDRs Idiosyncratic Adverse Drug Reactions

IgE Immunoglobulin E

IL Interleukin

IP Intraperitoneal

ITAM Immunoreceptor tyrosine-based activation motifs

LAT Transmembrane adapter protein linker for the activation of T-cells

LC-MS/MS Liquid chromatography tandem mass spectrometry

LPS Lipopolysaccharide

LST lymphocyte stimulation test

LPT lymphocyte proliferation test

LTA Light transmission aggregometry
LTT lymphocyte transformation test
MAC Mono-N-acetyl-4-aminodiphenyl sulphone
MHC Major Histocompatibility complex
Mins Minutes
Mo-DC Monocyte-derived dendritic cells
MRM Multiple reaction monitoring
NHS National Health Service
NK Natural killer
PAMP Pathogen associated molecular pattern
PBMC Peripheral blood mononuclear cells
PBS Phosphate buffered saline
PCR Polymerase chain reaction
PE Phycoerythrin
pH Potential of hydrogen
PHA Phytohemagglutinin
pi Pharmacological interaction
pKa Acid dissociation constant
PKC Protein kinase C
PPV Positive predictive value
RIPA Radioimmunoprecipitation assay
RASTs Radioallergosorbent tests
RNA Ribonucleic acid
RPMI Roswell Park Memorial Institute
Rt Retention time
SD Sulfadiazine
SFC Spot forming cell
SI Stimulation index
SJS Stevens-Johnson syndrome
SLP-76 SH2 domain-containing leukocyte phospho-protein of 76kDa

SMX Sulfamethoxazole
SMX.NO Nitroso sulfamethoxazole
SMX.NOH Sulfamethoxazole hydroxylamine
SNSulphanilamide
STAT Signal Transducer and Activator of Transcription
SCH Sulfachloropyridazine
SX Sulfadoxin
SZ Sulfamerazine
TAP Transporter associated with antigen processing
TCCs T- cells clones
TCR T-cell receptor
TEN Toxic epidermal necrolysis
Th1 Type 1 helper cell
Th2 Type 2 helper cell
TNF- α Tumour necrosis factor- α
Tregs Regulatory T cells
TT Tetanus toxoid
UK United Kingdom

Publications:

Alzahrani, A., Ogese, M., Meng, X., Waddington, J., Tailor, A., Farrell, J., Maggs, J., Betts, C., Park, B. and Naisbitt, D. (2017). Dapsone and Nitroso Dapsone Activation of Naïve T-Cells from Healthy Donors. *Chemical Research in Toxicology*, **30**(12), pp.2174-2186.

Abstract:

Dapsone (DDS) is an antibiotic associated with hypersensitivity reactions in 0.5 to 3.6 % of patients. Although clinical diagnosis is indicative of a drug hypersensitivity reaction, studies have not been performed to define whether dapsone or a metabolite activates specific T-cells. Thus, the aims of this study were to (i) explore the immunogenicity DDS and nitroso DDS (DDS-NO) using PBMC from healthy donors and splenocytes from mice, (ii) generate human T-cell clones to characterize mechanisms of T-cell activation and (iii) explore how the two antigenic forms of the dapsone interact with HLA-B*13:01, which is associated with the development of the DDS hypersensitivity among patients with leprosy. Naïve T-cell priming to DDS and DDS-NO was successful in three human donors. DDS-specific CD4⁺ T-cell clones generated from 2 donors were stimulated to proliferate in response to the drug via a MHC class II restricted direct binding interaction. Cross reactivity with DDS-NO, DDS-analogues and sulfonamides was not observed. DDS-NO clones were CD4⁺ and CD8⁺, MHC class II and I restricted, respectively, and activated via a pathway dependent on covalent binding and antigen processing. DDS and DDS-NO-specific clones secreted a mixture of Th1 and Th2 cytokines, but not granzyme-B. Splenocytes from mice immunized with DDS-NO were stimulated to proliferate *in vitro* with the nitroso metabolite, but not DDS. In contrast, immunization with DDS did not activate T-cells. To explore whether similar T-cell responses are detected in hypersensitive patients expressing HLA-B*13:01, we obtained samples from 6 patients, and evaluated T-cell specificity and the phenotype of drug-specific clones. Lymphocytes from certain patients proliferated in the presence of DDS and DDS-NO. DDS and DDS-NO-specific CD4⁺ clones were generated: DDS-specific clones were activated by the drug binding directly to HLA molecules on antigen presenting cells, while DDS-NO-specific clones were activated via a hapten mechanism involving formation of drug protein adducts and antigen processing by antigen presenting cells. Mass spectrometry was used to show that DDS-NO modified cysteine residues on mouse serum albumin and human glutathione-S-transferase P1.

Collectively, these data show that DDS- and DDS-NO-specific T-cell responses are readily detectable in healthy human donors and hypersensitive patients, whereas in murine systems, only DDS-NO activates T-cells. Additional studies are required to explore how the two antigenic forms of DDS interact with HLA-B*13:01, which is associated with the development of the DDS hypersensitivity among human patients with leprosy.

Chapter 1: General introduction

Table of CONTENTS

<u>1.1 Adverse Drug Reactions(ADRS)</u>	2
<u>1.1.1 Importance of ADRs</u>	2
<u>1.1.2 Classification of Adverse Drug Reactions</u>	3
<u>1.1.3 Epidemiology of Adverse Drug Reactions</u>	5
<u>1.1.4 Clinical and economic impact of adverse drug reactions</u>	7
<u>1.1.5 Drug Hypersensitivity Reactions</u>	8
<u>1.1.6 Time Course of Drug Hypersensitivity Reactions</u>	9
<u>1.1.7 Classifications of Hypersensitivity Reactions</u>	10
<u>1.1.8 Diagnosis of Drug Hypersensitivity Reactions</u>	14
<u>1.1.8.1 Lymphocyte Transformation Test (LTT)</u>	17
<u>1.1.8.2 Lymphocyte Toxicity Assay (LTA)</u>	19
<u>1.1.8.3 ELIsport</u>	19
<u>1.1.8.4 Flow Cytometry</u>	21
<u>1.1.8.5 T-cell Cloning</u>	21
<u>1.1.9 Cutaneous Drug Reactions</u>	21
<u>1.2 Immune System</u>	26
<u>1.2.1 Innate Immune</u>	27
<u>1.2.2 Cellular Components of the Innate immune System</u>	27
<u>1.2.3 Natural Killer Cells</u>	27
<u>1.2.4 Adaptive Immunity</u>	29
<u>1.2.5 Cellular Components Of The Adaptive Immune System</u>	30
<u>1.3 Mechanisms Of Drug Specific T- Lymphocyte Activation</u>	34
<u>1.3.1 Hapten Hypothesis</u>	38
<u>1.3.2 Pharmacological Interaction Of Drugs With Immune Receptors (PI Concept)</u>	40
<u>1.3.3 Altered Self-Peptide Repertoire Model</u>	41
<u>1.3.4 Danger Hypothesis</u>	41
<u>1.4 Xenobiotic And Drug Metabolism</u>	43
<u>1.4.1 Drug Metabolism Enzymes</u>	43
<u>1.5 Drug Metabolism Pathways</u>	47
<u>1.5.1 Phase I Drug Metabolism Reactions (Functionalization)</u>	47
<u>1.5.2 Phase II Drug Metabolism Reactions (Conjugation Reactions)</u>	48
<u>1.6 Dapsone</u>	49

1.1 Adverse Drug Reactions

Drugs have therapeutic properties, and often they are accompanied with undesirable effects known as adverse drug reactions (ADRs). These reactions have been present since the evolution of modern day pharma and the drug development process and are considered as a large public health issue throughout the world. An ADR is defined as “an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product.” (Edwards and Aronson, 2000).

1.1.1 Importance of ADRs

ADRs have great influence on health care systems and are a major concern for health care providers as it has been reported that 6-7% of all hospital admissions are associated with ADRs (Gomes and Demoly, 2005). Furthermore, ADRs are associated with significant patient morbidity and a high mortality rate (Pirmohamed et al., 1998, Classen et al., 1997, Ring and Brockow, 2002). The incidence of ADRs in Western countries is between 0.15% to 0.41% (Lazarou et al., 1998, Pirmohamed et al., 2004). They are the seventh most common cause of death (Wester et al., 2008). A study conducted in England found that there has been a 76.8 % increase in the annual number of ADRs and a 10% increase in mortality rate over the period of 10 years from 1999-2009 (Wu et al., 2010). The same increasing trend in mortality has been reported in the US (Shepherd et al., 2012). The ADR incidence in US is reported to be 6.7 % and fatal ADRs being 0.32% in hospital patients (Lazarou et al., 1998) . In the European Union, ADRs cause 197,000 deaths annually (Bouvy et al., 2015). In the pharmaceutical industry, an ADR is one of the most important cause of drug attrition (Daly, 2013).

Clinically, ADRs manifest as symptoms such as skin rash, urticaria, itching, fixed dose eruptions, angioedema, Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis

(TEN) in addition to numerous hematologic problem and GI disorders (Sharma and Sethuraman 1996; Sharma et al. 2001). The most affected organs are the liver and kidney (Arndt and Jick 1976; Hunziker et al. 1997; Chen et al. 2013)

1.1.2 Classification of Adverse Drug Reactions

There are different classification systems for grouping ADRs, but generally they are classified as Type A and type B reactions (Riedl and Casillas, 2003). The main differences are summarized in Table 1.1. These are also called dose dependent and idiosyncratic reactions respectively. This simple classification is based on dose dependence and predictability (Rawlins, 1981).

Type A reactions depend on the dose and because of the established pharmacology of the drug, they are predictable. These reactions are non-immunological in nature. Type A reactions are most common and account for 80% all drug reactions (Park and Coleman, 1988; Ritter, 2008). These reactions are caused by either pharmacological or toxic properties of a drug and may occur in anyone (Rieder, 1994).

Type B reactions do not depend on the dose of the drug and are for the most part non-predictable. Studies have shown that 10-15 % of all reactions are Type B (Jick, 1984). There is variation in literature studies about the classification of Type B reactions and the use of various terms. Most of the scholars classify type B reactions into two types (a) immune mediated and (b) non immune mediated. 5 to 10% of all drug reactions involve activation of the host adaptive immune system and are referred to as immune mediated which include hypersensitivity reactions and IgE mediated allergic reactions (1999, deShazo and Kemp, 1997, Anderson and Adkinson, 1987). They involve different components of the adaptive immune response including IgE antibodies, drug-specific T-cells and immune complexes (deShazo and Kemp, 1997, Thong and Tan, 2011) which will be discussed in detail in this thesis. The remaining non-immune mediated Type B reactions are classified into various types such as as

pseudo-allergic, idiosyncratic and intolerance (Riedl and Casillas, 2003). Others have used the term idiosyncratic to describe Type B reactions, which can be further subdivided into immune mediated and non-immune mediated reactions (Aberer et al., 2003, Pichler, 2003).

It is not possible to put all drug reactions into type A and B because of the complications so scholars extended this system with more types, also labelled alphabetically. These types are Type C, D, E, F, and G. Under the Group C, reactions are classified based on biological characteristics dependent upon a drug's chemical structure. The Group D reactions are associated with delayed type reactions. The Group E deals with drug administration programmes with a particular focus on the 'end of treatment' effects. The Group F has to do with therapy failure and describes the adverse behaviour of the drug. The Group G is primarily focused on predisposed genetic reactions (Ritter, 2008).

Table 1.1 General classification of ADRs

Characteristics	Type A	Type B
Dose Dependency	Dose related	Dose relationship not defined
Adverse reactions	common	uncommon
Reaction severity	Variable, usually mild	Variable, usually severe
Genetic factors	Abacavir HLA-B*57:01 and carbamazepine-HLA-B*15:02	May be correlated
Animal model	Known to reproduce in animal models	Unknown to reproduce in animal models
Frequency	80%	20%
Predictable from Known pharmacology	Yes	Not usually
Frist detection	In clinical studies	Occasionally in phase III-IV
Clinical burden	High morbidity & low mortality	High morbidity & High mortality

1.1.3 Epidemiology of Adverse Drug Reactions

The epidemiology of ADRs has been a major focus for scholars and clinicians for the last 50 years because of their complications. In most cases, the total number of individuals affected with ADR in different regions of the world are not known because of underreporting. For example one study found that underreporting of ADRs can be as high as 94% (Hazell and Shakir, 2006). Although ADRs are a global problem, there is high variability in data across the world. It has been found that 5 % of all hospital admissions in Europe are caused by ADRs (Bouvy et al., 2015). In USA and Canada ADRs account for 4.2-30% of hospital admissions, and 5.7-18.8% of admissions in Australia (Howard et al., 2007). Jemal at al (2005) suggest

that ADRs are the fourth leading cause of death in USA coming after heart disease, cancer and stroke (Jemal et al., 2005) and the annual death toll is 106,000 (Lazarou et al., 1998). Another study in USA found that between 3% and 6% of ADRs are fatal or have serious concerns, with an assessed 140,000 fatalities secondary to ADRs happening yearly in the USA (Wester et al., 2008, Lazarou et al., 1998). Ethnicity and advances in health care medical practices are some of the factors which cause variability in epidemiology data in various regions of the world (Raschetti et al., 1999; Fattinger et al., 2000; Davies et al., 2009).

There are a number of risk factors associated with ADRs. The factors are related to drug and patient while the rest are pathophysiological (**Fig 1.1**). Chemical properties, structure and molecular weight of the drug are one the most important drug related-risk factors associated with ADRs (Riedl and Casillas, 2003). Drugs with complex structure and high molecular weight are more likely to cause immunological ADR (Holt and Ju, 2006, Uetrecht, 2007). Additionally, the dose of drug administered is a vital susceptibility factor (Leach and Roy 1986; van der Ven et al. 1991). Regarding gender, females are more affected than males (Barranco and Lopez-Serrano, 1998, Haddi et al., 1990). Different diseases also make individuals susceptible to ADRs. For example patients with AIDS are 10 – 50 times more likely to develop ADRs (Coopman et al., 1993). Other risk factors reported in literature studies are rural residential location, socioeconomic status (Sikdar et al., 2012) cardiovascular disease, depression and diabetes mellitus (Naranjo et al., 1981, Hallas et al., 1990) impaired renal function (Conforti et al., 2012) and liver disease (Wawruch et al., 2009).

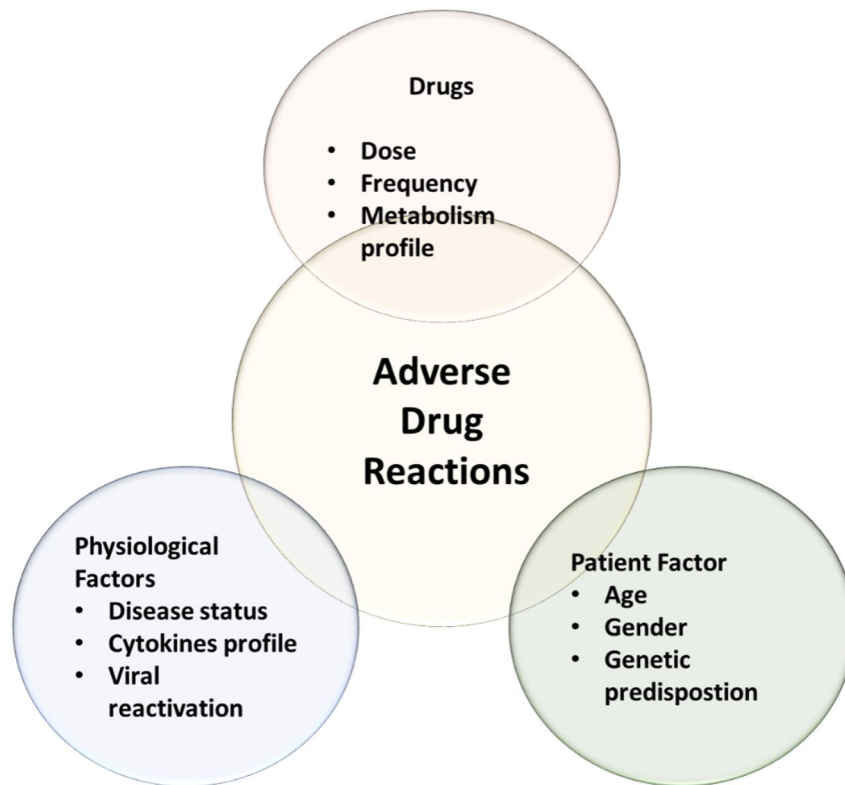


Figure 1.1 Risk factors associated with adverse drug reactions

1.1.4 Clinical and economic influence of adverse drug reactions

ADRs not only cause injury and death but they are also significantly associated with increased costs related to healthcare. There is a considerable burden of ADRs in both in- and out-patient settings (Bouvy et al., 2015). It has been suggested that 10% of healthcare costs are related to ADRs in hospitals (Moore et al., 1998). According to White et al and Patel et al the expected impact on hospital costs go above \$30 billion (White et al., 1999, Patel et al., 2007). A recent survey found that patients experiencing ADRs costs \$2284–5640 per head (Rodriguez-Monguio et al., 2003). In another survey done in Sweden, it was reported that individuals with ADRs had a 150% higher overall cost of illness compared to patients without ADRs (Gyllensten et al., 2013). The costs of ADRs in Intensive care unit are much higher than non-

Intensive care unit. For example a study done by Cullen et al found that ADRs in intensive care unit costs \$19,685 compared to \$13,994 in a non-Intensive care unit (Cullen et al., 1997). In the UK, extended hospital admission resulting from ADRs is estimated to be eight days and accounts for approximately 4% of the total capacity of hospital beds, costing the NHS about £466m (Pirmohamed et al., 2004). Moura et al. (2009) evaluated the occurrence of ADRs in the intensive care unit and assessed their effect on the staying period. Each ADR presented by the patient was correlated to a rise of 2.38 days in the intensive care unit (Moura et al., 2009).

The reasons which constitute the source of financial burden are increased hospitalization, prolongation of hospital stay and additional clinical investigations in more serious cases, and the out-patient care (Sultana et al., 2013, Field et al., 2005). Furthermore, there are also numerous secondary costs for patients and their care givers that are incurred by ADRs, such as missed days from work and/or morbidity such as anxiety due to the ADR episode (Wu and Pantaleo, 2003) Drug surveillance studies have found that ADRs such as fever, bleeding, diarrhoea and cardiac arrhythmia have great economic burden in health care setting (Classen et al., 1997).

1.1.5 Drug Hypersensitivity Reactions

There is not a single definition of drug hypersensitivity reaction. The terms “drug allergy,” “drug hypersensitivity,” and “drug reaction” are often used simultaneously. Drug hypersensitivity is defined as an immune-mediated response to a drug molecule in a sensitive patient while drug allergy is limited to a reaction mediated by IgE. Drug hypersensitivity reactions are also referred to as type B or idiosyncratic drug reactions (Park et al., 1998, Uetrecht, 1999). These reactions are difficult - to predicted from the known pharmacology of the drug. Hypersensitivity drug reactions represent up to one-third of adverse drug reactions (Bates et al., 1995, Lazarou et al., 1998, Pouyanne et al., 2000, Smith et al., 1996).

Gell and Coombs established their broadly accepted classification of these hypersensitivity reactions (Gell and Coombs, 1963). It has been widely accepted that drug hypersensitivity reactions are mediated by immunoglobulin (Ig) E or T cells (Johansson et al., 2001) and this has also been included in the Gell and Coombs classification (Pichler, 2003). More recently, the European Academy of Allergy and Clinical Immunology and the World Allergy Organization suggested a new and revised nomenclature to differentiate between allergic and non-allergic drug hypersensitivity Reactions (Johansson et al., 2001, Johansson et al., 2004).

1.1.6 Time Course of Drug Hypersensitive Reactions

To diagnose and treat drug hypersensitivity reactions it is very important to know the time course of the appearance of the clinical symptoms. Based on this clinically, drug hypersensitivity reactions are classified in to two types.

1. Immediate: reactions which occur less than one hour after the intake of the drug. **2. Delayed-type drug hypersensitivity reaction** which occur one hour to many days after using the medication. (Demoly et al., 2014). Delayed type are also classified as non-immediate drug reactions (Torres et al., 2003).

Immediate drug hypersensitivity reactions include IgE-mediated reactions presenting as urticaria, angioedema, bronchospasm, or anaphylaxis. Non-immediate drug hypersensitivity reactions, which are generally T cell-mediated, include: urticarial, maculopapular, and more severe exanthema. However, it is now becoming apparent that adverse events affecting internal organs also fall under this definition of drug hypersensitivity, onset of clinical symptoms can take minutes, several hours or days after drug ingestion (Romano et al., 2004). These kinds of reactions are related to different immunological mechanisms (IgE or T cells), activated by the drug (Pichler, 2003, Torres et al., 2003, Blanca et al., 2009, Romano et al., 2011). As this classification has both mechanistic and clinical implications, some authors consider it

problematic to separate drug hypersensitivity reaction based only on a cut-off of 1 hour (Bircher and Scherer Hofmeier, 2012). Moreover, considering that the clinical history is often unreliable, it is difficult to establish exactly when the reaction started. In the case of nonsteroidal anti-inflammatory drugs (NSAIDs), a consensus has been reached on the classification of drug hypersensitivity reaction as acute (immediate to several hours) and delayed (>24 hours) (Romano et al., 2011) mainly because most reactions are not mediated by a specific immunological mechanism (Bircher and Scherer Hofmeier, 2012). Difficulties in clinical assessment rise when evaluating urticarial reactions, which can be immediate or delayed, consequently showing that the same clinical image may develop by different mechanisms (IgE, T cells, or even non-immunological mechanisms, such as those involved in NSAID hypersensitivity) (Cornejo-Garcia et al., 2009).

1.1.7 Classification of Hypersensitive Reactions

Gell and Coombs (Romano and Demoly, 2007) classified immune-mediated reactions into four types: type I reactions (immunoglobulin E mediated); type II reactions mediated (through cytotoxic mechanisms); type III reactions mediated by (immune complexes); and type IV (T-cell reactions). Type IV reactions are currently split based on the heterogeneity of T-cell function into Types IVa, IVb, IVc and IV d (Pichler, 2003, Posadas and Pichler, 2007). Type IV hypersensitivity reactions are mediated by cellular immune mechanisms. The 4 categories involve activation and recruitment of monocytes (IVa), eosinophils (IVb), cytotoxic CD4⁺ or CD8⁺ T cells (IVc), and neutrophils (IVd) (Pichler, 2003).

Type I hypersensitivity is an immediate immune reaction to an antigen (Sicherer and Leung, 2009). Mast cells and basophils perform a fundamental role in Type I hypersensitivity reactions. Following exposure to an antigen, mast cells and basophils undergo process called degranulation, where they discharge substances that promote inflammation. In general,

antigens binding to IgE molecules result in mast cells degranulation (Yamasaki and Saito, 2005). Following mast cell degranulation the released inflammatory mediators include histamine, proteoglycans, serine proteases, and leukotrienes (Yamasaki & Saito, 2005). The immediate secretions of these inflammatory mediators can cause hives, redness, and angioedema (swelling of the throat, eyelids, lips and tongue) in what is referred to as an anaphylactic reaction (Noone and Osguthorpe, 2003). Sometimes, the anaphylactic reaction could cause blocked airways and heart arrhythmias (Noone & Osguthorpe, 2003). Although mast cells play a critical role in immediate reactions, it should be noted that antigens also activate B and T lymphocytes promoting IgE antibody production and antibody clone switching respectively.

Type II hypersensitivities, also recognized as cytotoxic hypersensitivities, are infrequent reactions that are usually caused by IgG and IgM antibodies (Brostoff et al., 1991). One of the reasons for the occurrence of the Type II response is the presence of desired antigen on the surface of a host cell and these are reported in most of the autoimmune diseases, drug sensitivity reactions, and organ transplantations (Brostoff et al., 1991). The binding of IgG and IgM antibodies to host cells is the biochemical basis of type 2 reactions and this binding then form complexes, activate the complement pathway resulting in the removal of host cells (Brostoff et al., 1991; Kornbrust et al., 1989). B cells, antibodies, and cytokines are major mediators of Type 2 hypersensitivity. They work together to induce cell lysis and eventually death. For example, the binding of drugs to the red blood cells of the host can be recognized as foreign pathogen. This binding then mediates the proliferation of B cells which then secrete antibodies and activate the complement system. All these steps finally lead to cells lysis (Kornbrust et al., 1989).

Type III hypersensitivities are also mediated by IgG and IgM antibodies. Unlike a Type II response, Type III hypersensitivity is associated with responses to soluble antigens that are not

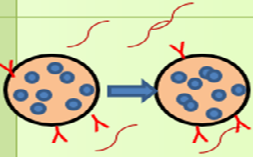

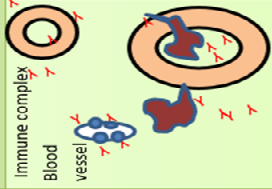
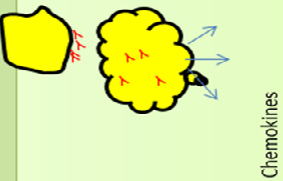
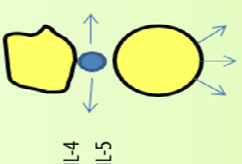
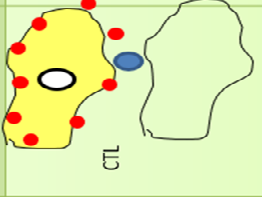
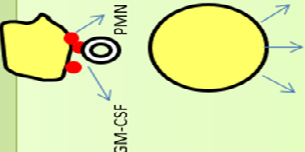
combined with host tissues but with antibodies in the blood which can then lead to inflammatory responses (Brostoff et al., 1991). The accumulation of antigen-antibody complexes in different parts of the body such as kidneys, skin and eyes induce an inflammatory response (Ellsworth et al., 2008). The reports have shown that Type III reactions are also involved in certain diseases such as systemic lupus erythematosus (SLE), serum sickness, and farmer's lung. A Type III reaction can take hours, days, sometimes weeks to develop (Coico & Sunshine, 2009).

Type IV hypersensitivities are considered to be delayed-type hypersensitivities because a reaction can naturally take one or more days to progress (Brostoff et al., 1991). Type IV responses are dependent on T cell interactions, which lead to recruitment of further cells to the site of exposure (Brostoff et al., 1991). Upon an initial antigen exposure, naive T-cells proliferate and specify into memory T cells after differentiation. Now these newly formed memory T-cells go into a dormant state until next contact with the same antigen. This provokes a quicker response and in response to new encounter with the same antigen, memory T cells instantly multiply and differentiate into the new types of cell called effector T cells. These cells then quickly eliminate the antigen or antigen bound cells. A common example of Type IV reaction is contact dermatitis. In this reaction lymphocyte proliferation produces local inflammation, causing a rash which characterise this condition (Nosbaum et al., 2009).

New studies have shown a new fifth type of hypersensitivity reaction. It is characterized by specific formations called granulomas which is a collection of macrophages and epitheloid cells, in the form of a round ball which encapsulate and isolate a pathogen (Rajan, 2003, Tercelj et al., 2008). Granulomas are formed in response to antigens that escape the primary phases of an immune response and are sometimes correlated to a delayed-type hypersensitivity (Tercelj et al., 2008). **Table 1.2** illustrates Gell and Coombs classification system.

Table 1.2 Gell and Coomb's classification system

Adapted from clinical aspects of immunology, textbook, 1975 (Coombs, Gell and Lachmann, 1975).

	Type I	Type II	Type III	Type IV a	Type IV b	Type IV c	Type IV d
IMMUNE REACTANT	IgE	IgG	IgG	INF- γ , TNF TH1 cells	IL-5, IL-4/IL13 TH2 cells	perforin granzymeB (CTL)	CXCL-B, IL-17 GM-CSF
ANTIGEN	soluble Antigen	Cell or matrix associated antigen	soluble antigen	Antigens presented by cells or direct t cell stimulation	Antigens presented by cells or direct t cell stimulation	Cell associated antigen or direct t cell stimulation	soluble Antigens presented by cells or direct t cell stimulation
EFFECTOR	mast cell Activation	FCR + cell	FCR+ cell	Macrophage activation	Eosinophil	T cells	Neutrophils
							
EXAMPLE OF HYPERSENSITIVITY REACTIONS	allergic rhinitis, asthma, systemic anaphylaxis	Hemolytic anemia thrombocytopenia	serum sickness, arthus reaction	Tuberculin reaction	Chronic asthma Chronic allergic rhinitis	Maculopapular exanthema	AGEP Behcet disease

1.1.8 Diagnosis of Drug Hypersensitive Reactions

It has been difficult to establish a diagnosis of drug hypersensitive reactions due to its variable clinical presentation, overlap with other clinical conditions and the often delayed temporal relationship between administration of the culprit drug and the appearance of symptoms (Knowles et al., 2000). Lack of a reliable and safe diagnostic test plays a major role in the significant morbidity and mortality due to drug hypersensitivity (Neuman et al., 2000, Choquet-Kastylevsky et al., 2001).

Diagnosis of non-immediate drug hypersensitivity reactions is complex and is usually made once the reaction has disappeared. The allergology examination including clinical profile history, skin tests, and drug provocation tests help to recognize the immunological mechanisms involved and the drug(s) responsible. In clinical practice, an accurate clinical history including the chronology of symptoms is necessary before selecting appropriate diagnostic tests. However, the clinical history is particularly difficult in patients with non-immediate drug hypersensitivity reaction (Aberer et al., 2003). A number of *in vitro* diagnostic tests have been used to aid the diagnosis of delayed-type drug hypersensitivity reactions (Primeau and Adkinson, 2001, Romano and Demoly, 2007, Naranjo et al., 1992, Lan et al., 2006). Among these tests are those that apply peripheral blood mononuclear cells (PBMC) as target cells, including the lymphocyte transformation test (LTT) and Light transmission aggregometry (LTA). Skin prick testing, intradermal tests with delayed reading, and patch testing have been commonly used for diagnosis. Intradermal tests are frequently optional when a dosage form of drugs are injectable form; patch tests are suggested when drugs cannot be diluted. However, the sensitivity of skin tests in non-immediate reactions is somewhat low, especially in children (Caubet et al., 2011, Padial et al., 2008). Hence, the drug provocation test is regularly the only test available, in spite of its inherent risk, it's time-consuming, and hence associated with significant cost, contraindicated in severe reactions such as bullous eruptions or desquamative

exanthema. In addition, the interval between drug administration and development of symptoms ranges from more than a 1 hour to several days, and the distribution and extension of skin manifestations can complicate evaluation of the results (Padial et al., 2008).

Provocation testing also known as immediate-type skin testing is a traditional method to diagnose Type I hypersensitivity reactions. In this method, certain topical antigens are challenged and later dermal response of patient is noted. (Smith, 1992). Clinically, provocation testing can be dangerous because certain antigens can cause a severe anaphylactic reaction (Smith, 1992). An alternative method to provocation testing is skin testing which involves skin pricks or patches to determine hypersensitivity. In prick test and scratch test, skin is pricked with a needle or pin. The needles contains very small amount of certain antigen whereas in s patch test a patch containing known antigens is applied to the skin (Williams et al., 1992). The presence of redness or swelling at the site of prick or patch is considered a positive diagnosis (Williams et al., 1992). Another way to diagnose Type I hypersensitivity is the radioallergosorbent tests (RASTs). The amount of IgE antibody that reacts to specific allergens is detected in the RAST (Primeau and Adkinson, 2001); Williams et al., 1992). If this test shows high level of IgE to a specific antigen, then that person is considered to be allergic to that antigen (Primeau & Adkinson, Jr., 2001). Leukocyte histamine release assays, surface markers for basophil activation, and leukotriene release tests are other important tests to detect Type I hypersensitivity reactions (Primeau & Adkinson, Jr., 2001).

Type II hypersensitivity reactions are mediated by IgM and IgG antibody responses to host tissues. For instance, Goodpasture syndrome is an autoimmune disease characterized by inflammation of the glomeruli in the kidneys and hemorrhaging of the lungs (Salama et al., 2001). Good pasture syndrome is diagnosed by measuring IgG antibodies to glomerular basement membrane (anti-GBM) (Salama et al., 2001). Another important test is the anti-neutrophil cytoplasmic IgG antibodies (ANCA) test which is used to detect various

Chapter 1

autoimmune disorders that may be connected with Type II hypersensitivities (Radice and Sinico, 2005).

Transplant and blood transfusion patients are mostly prone to Type II reactions and in these cases allergy is determined by signs and symptoms of patient or by tissue biopsy (Stapel et al., 2008).

Although limited data exists about methods for the detection of Type III hypersensitivity reactions, some suggest serum IgG antibody testing can be utilized (Stapel et al., 2008). Recent literature on food sensitivities recommends that the most important assay to detect IgG antibodies to specific dietary proteins is the enzyme-linked immunosorbent assays (ELISA). An increased quantity of IgG antibodies to a specific dietary protein was found to be diagnostic for a Type III hypersensitivity to that protein. Cessation from eating the reactive food allergen in that case would therefore be recommended. However, further research is necessary to confirm this test as a reliable method to diagnose Type III hypersensitivities to various antigens including drugs (Scott et al., 1990).

For the detection of type IV hypersensitivities a number of methods have been developed and modified. Although, the role of T cells in hypersensitivity reactions has not received good attention in the past, advances in recent research has elucidated and investigated the role of T cells in hypersensitivity and it has helped a lot in understanding of delayed-type reactions (Primeau & Adkinson, Jr., 2001). This has resulted in the more widespread use of testing for diagnosis, for the detection and verification of Type IV hypersensitivity in patients (Primeau & Adkinson, Jr., 2001).

There is much similarity in delayed skin testing and immediate-type skin test, except that delayed testing is read after 24 or 72 hours rather than 15 minutes after application of the antigens to skin (Li, 2002). There is a great chance the skin testing would pretense the danger

of developing adverse systemic reactions therefore proper safety should be ensured while doing these tests. (Reid et al., 1993). Although skin testing has been used for a long time and have proved reliable in several cases it can still be unreliable in certain cases (Sampson and Albergo, 1984). Due to the low sensitivity of skin testing particularly with several drugs alternative *in vitro* procedures have now been developed for the detection of Type IV hypersensitivities including methods such as the Lymphocyte Transformation Test LTT (Pichler and Tilch, 2004).

The *in vitro* study of the cell response in non-immediate reactions mainly assesses the T-cell response. A cellular response relating drug-specific-cell activation could be evaluated *in vitro* by measuring T-cell proliferation (Pichler & Tilch, 2004). However, the readout for T-cell activation can be changed, for example, flow cytometric lymphocyte activation test, which detects upregulation of the activation marker CD69 (Beeler et al., 2008). Furthermore, flow cytometry, ELISA and ELI spot can be used to detect drug-specific cytokine release (e.g., IFN- γ , IL-2, IL-5, IL-8 and IL-12) and the process of these assays are discussed in more detail below.

1.1.8.1. Lymphocyte Transformation Test (LTT)

The terms lymphocyte transformation test (LTT), lymphocyte stimulation test (LST) and lymphocyte proliferation test (LPT) are interchangeably used to describe this technique. The *in vitro* lymphocyte transformation phenomenon was first described during the late 1950s. In short, human peripheral leukocytes differentiate in short-term primary cultures, forming blasts. Phytohemagglutinin (PHA), of plant source from red kidney beans (*Phaseolus vulgaris*), was used in early studies to isolate blood peripheral leukocytes (Rigas and Osgood, 1955). PHA results in erythrocytes aggregation and sedimentation causing leukocytes to separate from whole blood preparations (Minor and Burnett, 1948, Li and Osgood, 1949).

Leukocytes are present in peripheral blood at densities of $5-7 \times 10^3$ cells/mm³; 20 to 50% of these cells are lymphocytes whereas 2 to 10% are monocytes. Lymphocytes are favoured as a model for investigation of immune-mediated diseases because of their unique characteristics, which include that (i) they are easily obtained at adequate density; (ii) they play a significant role in the immune system by orchestrating different elements of the immune response and thus representing the state of the immune system in the specific patient; (iii) they are metabolically active and expressing most of the enzymes that are required for drug activation and detoxication; and (iv) individual genetically-based faults in the expression or activity of these metabolic enzymes are phenotypically expressed in lymphocytes (Shear and Spielberg, 1988).

In the LTT PMBCs, isolated from drug hypersensitive patients and control subject are cultured with non-toxic concentrations of the drug implicated in the reaction. Any increase in the rate of cell proliferation measured by [³H] thymidine incorporation is a marker of T-cell activation. The increase in cell proliferation is expressed as a ratio between proliferation of cells incubated with and without the drug. This ratio is defined as the stimulation index (SI) and it is calculated as follows: proliferation due to drug divided by proliferation as result of medium control (Rigas and Osgood, 1955).

The LTT has been utilized by different investigators for diagnosis of prospective drug hypersensitivity for more than 20 years (Nyfeler and Pichler, 1997). The sensitivity and specificity of the LTT in diagnosis of drug allergy has been estimated to range from 56% to 78%. These estimates are generally based on cases of reaction to β -lactam antibiotics and cannot be extended to all other types of drugs (Luque et al., 2001, Nyfeler and Pichler, 1997). Moreover, the sensitivity is much lower when the assay is applied to GP diagnosed or self-diagnosed reactions, likely due to mis-diagnosis rather than a failure of the assay.

1.1.8.2 The lymphocyte toxicity assay (LTA)

Presented by Spielberg and colleagues (Spielberg, 1986, Spielberg, 1984, Spielberg, 1980) in the 1980s, the LTA is an *in vitro* test which uses isolated PBMC to explore the pathogenesis of idiosyncratic drug reactions. The test is based on the hypothesis that idiosyncratic reactions develop because of a difference between generation of toxic reactive metabolites (metabolic activation or toxication) and detoxication capacity which leads to increase of toxic metabolites (the “reactive metabolite” hypothesis) (Knowles et al., 2000, Uetrecht, 2007, Uetrecht, 2008). In general, the test does not use lymphocytes as immunogenic cells but rather as surrogate target cells (Spielberg, 1980). It is based on incubation of PBMC isolated from the patient with the hypersensitive drug in the presence of phenobarbital-induced mouse, rat or rabbit liver microsomal 9,000 x g supernatant fraction (S9), as a source of cytochrome P450 (CYP) monooxygenase activity (Uetrecht, 2008). An increased killing of lymphocytes by the generated metabolites, when compared with cells from tolerant controls is taken as diagnosis of hypersensitivity.

1.1.8.3 Enzyme-Linked Immunospot Assay (ELISPOT)

Determination of cytokines is a promising *in vitro* readout system in the diagnosis of drug hypersensitivity reactions (Porebski et al., 2011). Production after T-cell activation, which occurs at 24-48 hours, could also make the incubation time shorter than that required for cell proliferation in the LTT while conserving its advantages. The ELISPOT assay determines the increase in the number of cells producing a specific cytokine after their activation. This technique, which is similar to a conventional ELISA, is based on detection of the cytokine by a plate-immobilized specific antibody and identification by an enzymatically labeled secondary specific cytokine-antibody (Czerkinsky et al., 1988). The resulting plate contains a number of spots, each corresponding to a different single cell-secreting cytokine or cytotoxic marker and, in theory, any other secreted molecule (Rozières et al., 2009b, Zawodniak et al., 2010).

ELISPOT is highly sensitive in certain subjects and can detect fewer than 25 secreting cells per million peripheral blood mononuclear cells. ELISPOT has been used to detect lymphocytes secreting cytokines such as IFN- γ , IL-5, or IL-13 from allergic patients in the presence of the culprit drug. Recently, this test was also utilized to assess the cytotoxic response in drug hypersensitivity reaction by determining the release of granule content (granzymes and perforin) and cytokines (IFN- γ) by cytotoxic cells after activation with the hypersensitive drug (Rozieres et al., 2009b, Zawodniak et al., 2010). The test showed high sensitivity and specificity, although in some cases results did not correlate with the LTT, probably because cytotoxicity-based tests measure effector cell function, which is different to the proliferative response, where the cell subpopulation activated may be heterogeneous. (Rozieres et al., 2009b).

A recent study relating ELISPOT with skin testing in the diagnosis of cephalosporin-induced maculopapular exanthems (MPE) revealed that measuring both IFN- γ and IL-5 is more sensitive than skin testing for the diagnosis of cephalosporin hypersensitivity (Tanvarasethee et al., 2013). Therefore, quantification of cytokines such as IL-2, IL-5, IL-13, and IFN- γ is a promising diagnostic tool in most drug hypersensitivity reactions, although further studies are needed with larger series of hypersensitive patients and controls to evaluate the sensitivity and specificity of the technique, together with appropriate cutoffs to be used. (Porebski et al., 2011).

1.1.8.4 Flow cytometry

Flow cytometric analysis of intracellular cytokines has been used as a general scientific application in many cell types in domains such as immunology and rheumatology (Schuerwegh et al., 2003). In the study by Martin et al, IL-5/IL-10/IFN- γ measurement with flow cytometry and IL-5/IL-2/ IFN- γ with ELISA were evaluated in the PBMC of 19 patients after stimulation with the hypersensitive drug (Martin et al., 2010). Drug hypersensitivity was proved by stringent history taking. Drug-specific cytokine production by peripheral blood mononuclear cells was confirmed in 75% of the patients using flow cytometry and in 79% using ELISA, respectively. The combination of flow cytometry and ELISA increased the sensitivity to 100%. Other comprehensive studies are required to consider whether a specific-cytokine or combination of cytokines is best suited for the diagnosis of drug hypersensitivity. (Nyfeler and Pichler, 1997).

1.1.8.5 T- cell cloning

T cell cloning is used to determine the involvement of drug specific T lymphocytes in hypersensitive patients (Zanni et al., 1998). It is highly valuable tool for studying the way in which T cells recognise drugs and the function exerted by drug specific T cells *in vivo* on drug specific stimulation (Pichler et al., 1998). Using the well-established method of serial dilution, single T-cells can be expanded and antigen specificity assessed (Mauri-Hellweg et al., 1995; Staszewski, 1984). T-cell clones can then be fully characterised in terms of phenotype, for the expression of specific markers, proliferative capacity and their cytokine secretion profile. These studies help to discover the patho-mechanism of drug hypersensitivity.

1.1.9 Cutaneous Drug Reactions

In non-immediate drug hypersensitivity reaction, the skin is most commonly involved; other sites might not be included (Caubet et al., 2011). The clinical appearances are different

depending on the immunological mechanism involved, although maculopapular exanthema (MPE) and delayed urticaria are the most recurrent reactions observed (Fernandez et al., 2009, Metz and Maurer, 2009). Other, more severe reactions include acute generalized exanthematous pustulosis (AGEP), drug reaction with eosinophilia and systemic symptoms (DRESS), bullous eruptions such as erythema multiforme, Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN) (Blanca et al., 2000, Ramirez-Gonzalez et al., 2009), fixed drug eruption (FDE), contact dermatitis (Bos and Kapsenberg, 1993), and serum sickness-like syndrome (Naisbitt et al., 2007).

The main reason why a drug administered orally or parenterally affects the skin is not known, although immunological mechanisms and/or the drug metabolism capacity of cutaneous cellular components may be involved (Blanca et al., 2000, Naisbitt et al., 2007). The skin was traditionally thought to be no more than a physical and biochemical barrier protecting the organism from outside agents, but in the last decade it has been revealed to have a role in the immune response. The cellular composition of the skin includes mast cells, macrophages, dermal dendritic cells, keratinocytes, and Langerhans cells, which are capable of producing cytokines that enhance recruitment of other cells that are part of the dynamic component (Metz and Maurer, 2009, Ramirez-Gonzalez et al., 2009). These cells include antigen presenting cells (APC), such as Langerhans cells, dendritic cells, monocytes, and macrophages, as well as T lymphocytes expressing skin-homing receptors, such as the cutaneous lymphocyte antigen (CLA) and various chemokine receptors (eg, CCR10, CCR4, CCR6), which constitute the cellular basis of the immunological memory in the skin (Fernandez et al., 2009, Blanca et al., 2000, Bos and Kapsenberg, 1993).

1.1.9.1 Maculopapular Exanthema (MPE)

MPE consists of diffuse cutaneous erythema with areas of skin elevation. MPE may evolve to become vesicular or papular as part of a more severe clinical entity. There is also a possibility that different degrees of angioedema with involvement of subcutaneous tissue may also appear. Few studies have been carried out over the last 10 years on the mechanisms of other identities with the exception of drug allergy. MPE reactions are commonly caused by drugs, various viruses with skin tropism, such as cytomegalovirus, parvovirus, Coxsackie's virus, paramyxovirus, toga virus and several types of herpes, such as varicella zoster, HHV6 and the gamma herpes virus of infectious mononucleosis. MPE can also be observed in different bacterial diseases, in patients with lymphoma, in graft versus host disease and in diseases with other causes, such as physical agents. Further studies from Fernandez and his colleagues (2009) have shown that cytokine patterns that present themselves in maculopapular exanthema have a Th1 or Th0 pattern in accordance with whether the reaction is drug-induced or virus-induced and whether CD4⁺ T-lymphocytes having cytotoxic capabilities are prevalent (Fernandez et al, 2009).

Usually accompanied by homogeneous redness of the skin with variable degrees of subcutaneous involvement including oedema, MPE consists of maculopapules of different sizes elevated over the skin. Skin lesions usually subside if the drug is stopped soon after the appearance of symptoms. Even in patients who are taking the drug for a long time, exanthema does not usually develop to more severe diseases although some scleroderma-like lesions that have the potential to be induced by skin fibrosis may appear. The MPE evolves to clearly distinguish vesicular lesions when the exanthema forms part of another disease. The most severe of these vascular lesions are those appearing in the complex Stevens–Johnson–syndrome. (Fernandez et al, 2009).

1.1.9.2 Acute Generalized Exanthematous Pustulosis (AGEP)

Acute generalized exanthematous pustulosis is a rare drug-induced skin reaction characterised by a sudden skin eruptions (Amante et al 2009). According to Sidoroff (2001) acute generalised exanthematous pustulosis is distinguished by many non-follicular sterile pustules occurring on a diffuse and edematous erythema particularly in the on the face. In addition, patients suffering from these symptoms may show signs of fever and have an elevated level of blood neutrophils. Onset of the condition has been shown to be extremely acute, with symptoms becoming prevalent following the intake of the drug. It should be noted however, that viral infections can also trigger onset of the disease. Upon successful diagnosis and discontinuation of the drug course, symptoms such as fever and the pustules normally disappear within two weeks. In rare cases if left untreated mortality is observed in 5% of patients (Sidoroff et al. 2001). The onset of acute generalised exanthematous pustulosis involved the drug-specific activation of IL-8 secreting T-lymphocytes that recruit neutrophils to the site of the reaction commonly caused by the ingestion of antibiotics such as aminopenicillins and sulfonamides (Sidoroff et al. 2007).

1.1.9.3 Drug Reaction with Eosinophilia and Systemic Symptoms (DRESS)

DRESS is also known as the drug hypersensitivity syndrome (Amante et al 2009). Patient with this condition may manifest with symptoms such as high fever, skin rashes, malaise, lymphadenopathy and possible damage to multiple internal organs. With respect to this syndrome, liver damage is most common and this can often lead to fulminant hepatitis. The recruitment of eosinophils which is mediated through T-cell derived IL-5 is characteristic of DRESS. A systemic immune response can be elicited by the causative drug. This can result to the reactivation of human herpes viruses, HHV-6 and HHV-7. According to Picard (2010), 76% of affected patients experienced a reactivation of the herpes virus when affected by an offending drug. (Amante et al 2009). The link between viral reactivation and symptoms in the

course of DRESS is often used as diagnostic procedure (Suzuki et al., 1998; Tohyama et al., 1998). However, the role of viral reactivation in the pathophysiology of the disease and if it is a concern of the clinical manifestations is not known. Reactivation of human herpes virus 6 (HHV-6) is not usually measured for many weeks after symptoms appeared (Shiohara et al., 2006). Therefore, it is possible that viral reactivation alter a late CD8⁺ T-cell reponse activation. Transient increases in virus-specific immunoglobulins against HHV-7, Epstein-Barr virus (EBV) and cytomegalovirus (CMV) have also been detected in patients with DRESS (Aihara et al., 2001; Descamps et al., 2003; Picard et al., 2010). Cells from skin lesions have been analysed and found to contain high levels of HHV-6 DNA and express viral antigen at an early stage (Suzuki et al., 1998). It would be likely that certain clinical manifestations result from virus-specific T-cells expansion (Shiohara et al., 2006). And it is therefore possible that drugs activate CD4 cells, while viruses activate CD8 cells and both contribute to the pathogenesis of the disease.

1.1.9.4 Stevens - Johnson syndrome (SJS)

Several uncommon but life-threatening cutaneous adverse reactions such as SJS and toxic epidermal necrolysis (TEN) are most frequently caused by drug exposure. Widespread epidermal necrosis, resulting in flaccid bullae with epidermal shedding and regular involvement of the mucous membrane characterize both of these diseases. Both diseases in general improve within several days after the withdrawal of the causative drug. However, it is not clear whether these diseases are distinct from each other or simply variants of a related disorder. Both the diseases are considered as belonging to such a category as they both involve severe epidermolytic effects cutaneous drug reactions that differ only by extent of skin detachment (Kim et al. 2014).

1.2 The Immune System

The organ system that decides whether or not an antigen is part of a growing pathogen population is the immune system. This system ensures the wellbeing of an organism by detecting the invaders and distinguishing them from healthy tissues of the body (O'Byrne and Dagleish 2001).

The ability of the immune system to distinguish between non-threatening agents and hostile invaders is primarily based on pattern recognition. Studies aimed at identification or mapping of pattern recognition receptors (PRRs) have led to identification of chemical structures which receptors can recognize and bind to. Classes of microorganisms which stimulate these chemical structures have also been identified. Following the discovery of Toll-like receptors (TLRs) in 1990s, these receptors have assumed a prime position in immunological research. It is now well known that when activated TLRs can trigger a rapid immune response. TLRs recognize a wide variety of molecules including glycoproteins, liposaccharides, and nucleic acids. Studies involving the adaptive immune system have revealed the role of T-cell receptors and B-cell receptors in the identification of characteristic short sequences of amino acids (or epitopes) which serve as antigen (Bewick et al 2009).

The immune system in organisms can be divided into two subgroups: (i) innate immune system, and (ii) the adaptive immune system. In the early stages of invasion, the innate immune system protects the body and in later stages protection is mediated by the adaptive immune system. The different components of adaptive and innate immune systems together protect the body against foreign entities. The adaptive immune system is more specific in response to antigens than the innate immune system (Market and Papavasiliou 2003).

1.2.1 The Innate Immune System

The innate immune system is the non-specific first line of defence in the body system. There are two main components of innate immune system: the humoral and the cell-mediated responses. The humoral component includes inflammatory mediators, cytokines, coagulation cascades and the complement system, while the cellular component includes phagocytes, eosinophils and killer cells (Kumar et al. 2011).

The key role of the innate immune system is to recruit the immune cells to the site of invasion. This is done by the production of various cytokines and the activation of the complement cascade in order to identify bacteria, activate cells and promote the clearance of antibody complexes or dead cells (Janeway et al. 2001). The innate immune system can also activate the adaptive immune system through the process known as antigen presentation (Janeway et al. 2001). This process starts by setting an initial signal with antigens stimulating innate immune system via, for example, Toll-like receptors (TLRs) on dendritic cells. The activated dendritic cells then act as antigen presenting cells (APCs) as they process antigens and present in the form of peptides antigens to T cells. (Pichler 2008).

1.2.2 Cellular Components of the innate immune System

Innate immune system is equipped with a number of cell types which help to initially eliminate the pathogens and further activate the adaptive immune system. These cells include neutrophils, eosinophils, basophils, mast cells, macrophages, monocytes, natural killer cells and dendritic cells. (Janeway et al. 2001).

Natural Killer Cells: Natural killer (NK) cells are a central component of the innate response. They release granulysin after activation and are believed to act in unison with cytotoxic T cells to initiate tissue damage in patients. NK cells respond rapidly after exposure to virally infected cells in the absence of MHC restriction and T-cell receptor signaling. In

recent times Schlapbach et al. (2011) demonstrated that NK cells expressing granulysin make up a significant quantity of the cellular infiltrate in many forms of cutaneous ADR. Therefore, it is possible that NK cells contribute toward the tissue injury in patients with drug hypersensitivity. (Tewary et al., 2010).

Granulocytes: These are also called polymorphonuclear leukocytes. A subcategory of white blood cells, these cells are characterized by the presence of granules in their cytoplasm. These cells include neutrophils, eosinophils, basophils and mast cells. The granules in these cells contain cytotoxic chemicals that digest the microorganisms. The most abundant granulocyte are neutrophils. The cytokine IL-8 stimulates these cells and causes their migration towards the infected area. (Ovchinnikov, 2008)

Eosinophils: are important in parasitic infections. IL-5 and some exotoxins stimulate the eosinophils and they move to the site of infection or damage where they cause the destruction of parasitic pathogens with the help of toxic chemicals found in their granules. Within blood, basophils are the least abundant of granulocytes and their granular contents include histamine, heparin, chondroitin sulphate, and peroxidase enzymes as important constituents. Upon activation they cause the release of histamine which plays an important role in inflammatory response. (Ovchinnikov, 2008)

Macrophages: These are phagocytic cells. They engulf pathogens by the process known as phagocytosis and kill and digest them with the help of toxic chemicals present in their lysosomes. They also act as antigen presenting cells where they present processed antigens to helper T cells. Macrophages are characterized by the presence of CD14, CD40, CD11b, CD64 and CD68 markers and they are found in almost every tissue (Ovchinnikov, 2008). There are various activated forms of macrophages (Mosser and Edwards, 2008) and

they are divided broadly into two groups M1 and M2 based on different effect function (Galdiero et al. 2013).

Dendritic cells: Also known as accessory cells in traditional literature, dendritic cells are professional antigen presenting cells and are important components of the immune system. They are found in two distinct forms in the body, as mature and immature forms (Mellman and Steinman, 2001). Immature dendritic cells are mostly found in peripheral tissues and they are poor presenters of antigens to T cells even if they express both MHC I and MHC II. Upon activation by pro-inflammatory cytokines or pathogen-derived TLR ligands, dendritic cells mature and migrate to the T cell rich lymphoid organs where they stimulate memory and naïve T cells responses through much more efficient antigen presentation (Hammer and Ma 2013).

1.2.4 The Adaptive Immune System

When the body is confronted with pathogenic infection or injury, both the innate and adaptive immune systems become active in order to maintain homeostasis. These defence systems monitor tissues of the vertebrate body by using complex mechanisms to identify the threats and take measures to overcome those threats. The two types of defences differ in their arsenals and mechanisms. The adaptive immune system aims at prolonged protection over a period of time and with great specificity against the danger. Both means of defence have some coordination in order to protect against over expression of defence reaction by enhanced inflammation. The immune system has been described as an outcome of evolutionary process which distinguished ‘infectious non-self’ from ‘non-infectious self’. This paradigm can be used to devise a classification system comprised of adaptive immune responses of five different types. Nucleotide-binding oligomerization domain containing receptors or NOD-like receptors (NLRs) regulate their responses to these types based on the origin of target antigen: (i) antimicrobial immunity, where the target comprise of pathogens antigens, (ii) allergies,

involving targets from non-pathogenic sources, (iii) autoimmunity, involving targets within the self, (iv) tumor immunity, involving altered self-targets, (v) commensal homeostasis, involving foreign self-targets. In each of these response categories, the role of NLRs is different. Due to the difference in the source of initiation of immunity, the host response is also different. (Charles Janeway 2001). The adaptive immune system, also known as the acquired or specific immune system, is a subsystem of the overall immune system that acts to ensure a much faster response with regards to the interaction with antigen based on immunological memory. Its main function is to destroy invasive pathogens and their toxic products. Cellular and molecular interactions occur between the innate immune system and the adaptive immune system which allows the innate immune system to activate adaptive immune responses. The adaptive immune system differs from the innate immune system in that its responses are highly specific with regards to pathogens, with the adaptive immune system capable of providing long-lasting protection against disorders. The majority of responses within the adaptive immune system are mediated by B and T-lymphocytes, with B-cells being activated in reactions to secrete antibodies that will travel through the bloodstream, binding to foreign agents, thereby rendering them inactive. The adaptive immune response can be divided further into a cellular (cell-mediated) response or a humoral (antibody-mediated) response. Cell-mediated immunity is by induced cytotoxic T-lymphocytes that lead to apoptosis of target cells. Humoral immunity can be mediated by B-cell production of antigen-specific antibodies (Alberts et al. 2002).

1.2.5 Cellular Components of the Adaptive Immune System

T Lymphocytes. Naive CD4⁺ T lymphocytes differentiate into Th1, Th2, Th9, Th17, or Th22 effector cells after antigen exposure (Fig 1.2). The panel of cytokines naïve cells are exposed to at the time of priming determines the nature of the effector T-cell response and the functional consequences of antigen exposure (Akdis and Akdis, 2009). The classification of CD8⁺ T-cells is much simpler, based on the release of cytolytic molecules (Fas ligand, perforin,

granzyme B, granulysin) after antigen stimulation. Cutaneous drug reactions have been classified according to the phenotype of drug responsive T-cells isolated from peripheral blood of sensitive patients and the cytokine secretion profile (Pichler, 2003). Keratinocyte damage in patients with maculopapular reactions involves CD4⁺ and CD8⁺ T cells, and Th1 and Th2 cytokine release is readily demonstrable (Kuechler et al., 2004, Yawalkar and Pichler, 2001, Rozieres et al., 2009a). IFN- γ -secreting cytotoxic CD8⁺ T cells dominate in bullous skin reactions and DRESS (Naisbitt et al., 2003; Nassif et al., 2004; Ko et al., 2011, Wu et al., 2007). IL-5, which is implicated in eosinophil recruitment and activation, is determinable in drug-stimulated T-cell cultures from patients with DRESS. Pustular reactions involve CD8⁺-mediated cytotoxicity and secretion of the neutrophil chemoattractant IL-8 (Britschgi et al., 2001). Chung et al., 2008 showed that granulysin is a critical cytotoxic molecule released from T cells in patients with Stevens-Johnson syndrome/toxic epidermal necrolysis and proposed that high expression of granulysin in this group of patients could be the reason severe reactions develop. On the other hand, a latest study shows that granulysin is secreted from drug-specific T cells isolated from patients with mild, moderate, and severe cutaneous reactions (Schlapbach et al., 2011). However, it should be noted that, this classification is basically based on a snapshot of the memory T-cell response, frequently many years after the clinical reaction decreases. Further studies are required to compare the nature of the T-cell response at the time of drug exposure, during the hypersensitivity reaction, and in the long period of treatment, as the patient recovers (Naisbitt et al., 2003).

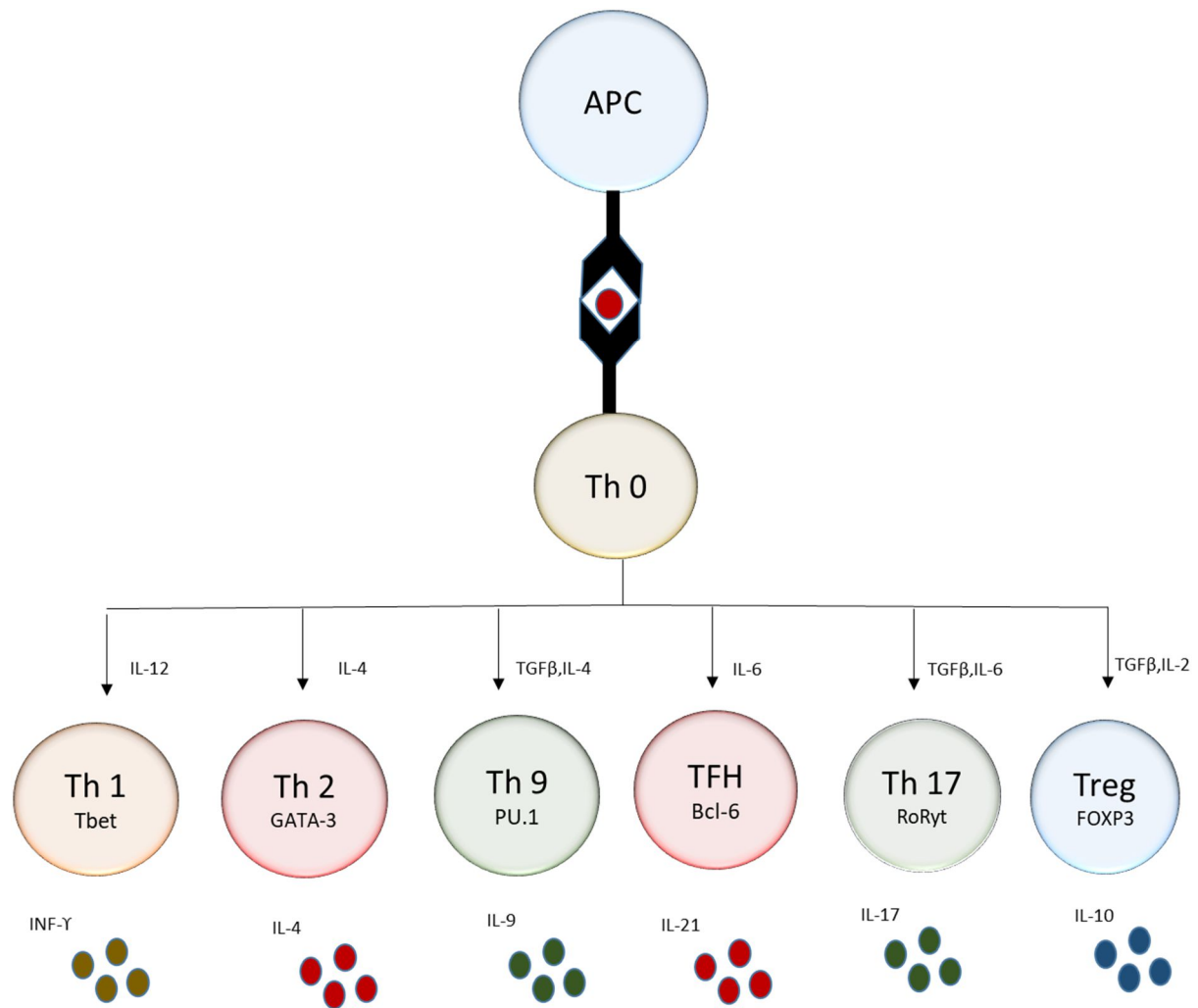


Figure 1.2 The overall development of T-cells from naïve CD4⁺ T-cells. CD4⁺T cells show remarkable plasticity and are able to differentiate into many different subsets based on the soluble molecules secreted during priming of the subsets by antigen presenting cells (APC), e.g., IL-12 for TH1 cells. The different subsets can be distinguished by the transcription factors that regulate and maintain their lineage-specific effector functions, e.g., T-bet for TH1 cells. The molecules secreted by these subsets, e.g., IFN- γ for TH1 cells, are fine-tuned to control the pathogen that mediated the release of the specific molecules by the APC during activation of the naive cells into the various subsets.

The adaptive immune system is also implicated in Idiosyncratic Adverse Drug Reactions that target the liver. However, the role of T cells in drug-induced liver injury is not well documented. Histologic investigation of liver from a patient with sulfasalazine-induced liver injury showed an infiltration of granzyme-B secreting T lymphocytes (Mennicke et al., 2009). The phenotype and function of T cells from patients with flucloxacillin-induced liver injury have previously been investigated (Monshi et al., 2013). Flucloxacillin exposure is related to a high incidence of cholestatic liver injury. Peripheral blood mononuclear cell responses against the drug were detectable in 5/6 patients using an IFN- γ secretion (ELISPOT) assay. T-cell cloning revealed that the majority of flucloxacillin responsive T- cells were CD8+. Drug – specific T-cells secreted IFN- γ , and cytolytic molecules, including FAS ligand, perforin, and granzyme B (Monshi et al., 2013).

B Lymphocytes. Restimulation of antigen-specific memory B-cells leads to a rapid increase in serum antibodies. Antigen-specific B-cells also effectively present peptide fragments to specific T cells (Lanzavecchia, 2007). Enhanced antigen presentation is dependent on membrane-associated antibodies that sequester and concentrate the antigen prior to processing. Soluble antigen-bound antibodies have also been shown to modulate the presentation of peptide antigens to T-cells. The impact on the T-cell response by enhancing antigen capture and delivery by modulating processing pathways, hence suppressing the generation of main antigenic determinants and by preventing processing (Watts and Lanzavecchia, 1993). Many studies have utilised drug-protein conjugates to identify anti-drug antibodies in certain tolerant and allergic patients (de Haan et al., 1986, Christie et al., 1988, Daftarian et al., 1995, Torres et al., 1997). However, the dynamics of the drug antigen-specific humoral response and the kinetics of antibody production have not been defined (de Haan et al., 1986). It is now understood that IgG4 antibodies play a central role in immune regulation after grass pollen immunotherapy (James et al., 2011). It is believed that IgG4 antibodies are produced by B cells

under the regulation of allergen-specific regulatory T cells that appear during immunotherapy (Satoguina et al., 2008). Suppressive IgG4 antibodies are believed to directly inhibit facilitated antigen presentation to T lymphocytes. It is therefore important to reflect on the critical roles played by specific IgG antibody subclasses in drug reaction (van Neerven et al., 1999).

1.3 Mechanisms of Drug-Specific T-lymphocyte Activation

In drug hypersensitivity reactions, the pathomechanism(s) of T-cell activation is still not clear. Many drugs are not protein reactive and metabolic activation is needed to produce an electrophilic intermediate with the capacity to haptenate protein. Most of the drugs that cause a high incidence of hypersensitivity form reactive metabolites and there appears to be some association between the extent of metabolic activation and the potential that administration of a drug will be linked with hypersensitivity (Manchanda et al. 2002).

Antigen processing and presentation are key pathways in the activation of T-cells. During this process an antigen is ingested by an antigen-presenting cells (APC) and is digested into peptide fragments that are then displayed on the surface of cell by complexing with molecules called the major histocompatibility complex. Most of the cells in the body express MHC and can act as APC, but macrophages, lymphocytes and dendritic cells are considered professional antigen presenting cells.

The intracellular proteins are processed into shorter peptides by a number of enzymes in an organelle known as the proteasome (Meissner et al., 2010). The proteasome is a large organelle of the cell with complex structure consisting of a number of subunits. The two prominent subunits which play a critical role in the processing of the proteins are LMP2 (Large Multifunctional Protease-2) and LMP7 (Large Multifunctional Protease-7). These subunits are encoded within the MHC locus. One of the initial steps in degradation of proteins is the binding of the proteins with a rather smaller but specialised protein known as ubiquitin. Then this

Chapter 1

ubiquitin protein complex is targeted towards the proteasome for further processing. Once proteins interact with the proteasome, LMP2 and LMP7 subunits induce the proteolytic complex of proteasome to cut the proteins and generate smaller peptides. The resulting peptides bind to MHC-I molecules. The actual processing takes place in the specialized area of proteasome known as channel of proteasome. From proteasome the Peptides move towards cytosol and finally are transported into the RER (Rough Endoplasmic Reticulum). Inside ER the peptides interact with MHC molecules (Rizvi and Raghavan, 2010). This transportation is carried out by a transmembrane heterodimeric protein called TAP (Transporters Associated with Antigen Processing). The peptides which are present in RER and are ready to bind with MHC consist of 8–13 amino acids. A crucial protein known as tapasin tethers the MHC-I molecules to the TAP transporter, and peptides are then loaded onto the MHC-I molecule. In the final step the peptide and MHC1 complex is transported to the cell surface where CD8+ T-cells recognize and bind to it (Neefjes et al., 2011).

The mechanism of processing exogenous or extracellular antigens is a bit different. First, antigen presenting cells (APCs) such as macrophages, dendritic cells and B-cells internalize these extracellular antigens. This process of internalization is called phagocytosis or endocytosis. The antigens are then processed and are transported to the surface of cell in combination with MHC II molecules. (Jensen, 2007).

Once the antigens have been internalized they pass through three acidic endosomal environments step by step with each next step more acidic than the previous one. These steps are early endosomes (pH 6.0–6.5), late endosomes (pH 5.0–6.0) and lysosomes (pH 4.5–5.0). Hydrolytic enzymes are present in all three stages which work in acidic environment and degrade proteins into smaller peptides consisting of 13–18 amino acids. Class-II Alpha and

Chapter 1

Beta chains associate within the RER. A specialized protein known as the Ii (Invariant Chain) plays a key role in preventing endogenous peptides from binding to MHC II. This protein also plays a crucial role in many other functions including: 1. Folding of α and β chains, 2. Exiting the peptide MHC complex from the ER, 3. Targeting to the endocytic compartments. Other than that Ii also helps in transporting the Class-II heterodimer to the late endosomal compartments: MIICs (MHC-Class II Compartments) where peptides and MHC-II molecules are complexed with each other. Ii is then degraded by a specialized enzymes called proteases (Cathepsins). The end product of this degradation is small fragment called CLIP (Class II-associated Invariant Chain Peptide). These CLIPs keep attached with the peptide-binding cleft of MHCII and help to preventing the premature interaction and binding of partially processed antigens. Another vesicle membrane protein, known as HLA-DM removes the CLIP from peptide binding cleft so that new completely processed peptides could bind there. The MHC-II-peptide complex then moves to the plasma membrane where the neutral environment stabilizes it and presents the peptide to CD4⁺ T-cells (Costantino et al., 2012). Figure 1.3.

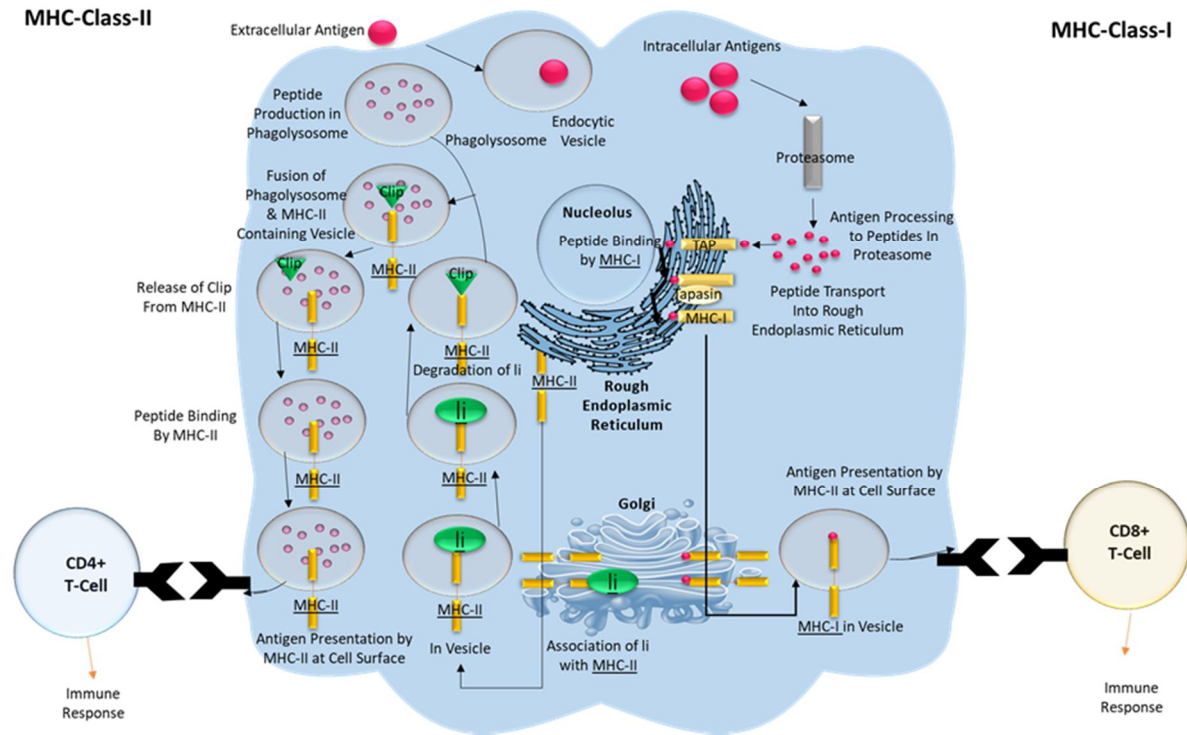


Figure 1.3 Antigen presentation Antigen processing is divided into two pathways. Extra-cellular and intra-cellular pathways. Extracellular antigens are engulfed by phagocytosis and degraded by endosomal enzymes. MHC class II molecules are transported in vesicles coming from the endoplasmic reticulum (ER) and Golgi apparatus. These vesicles fuse together with the phagolysosome and this is where loading of peptide occurs. The peptide bound MHC is then transported to the surface of the cell where the antigen is displayed. Intracellular antigens are broken down in the proteasome. Peptides are transported to the ER through TAP and attach to MHC class I molecules. The peptide bound MHC is then transported again to the Golgi where it is loaded into a vesicle and trafficked to the surface of the cell. Adapted from (Neefjes et al., 2011).

The three theories which explain the role of T-lymphocytes in the drug-specific activation and ADRs are the hapten theory, p-i concept and altered-self peptide concept. Figure1.4

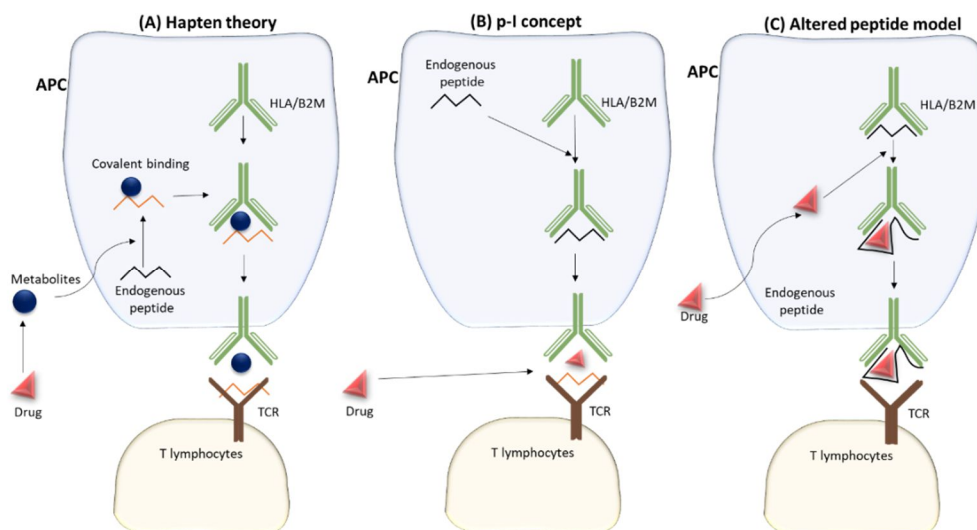


Figure 1.4 Pathways of drug-specific T-cell activation**A)** Hapten hypothesis where drug or metabolite binds covalently and modifies target proteins. Peptide fragments are presented by MHC molecule to the TCR on T-cells. Presentation through MHC I provokes a CD8⁺ T-cell response while MHC II presentation elicits a CD4⁺ T-cells response. Sometimes the drug or a metabolite modifies the MHC-peptide complex directly for presentation to T-cells. **B)** p-I hypothesis where drug or metabolite binds directly to the TCR and/or MHC molecule in a non-covalent way, so provoking an immune response. **C)** Altered peptide hypothesis where interaction of peptide with HLA molecule and TCR. Consequently, the presence of drug within the peptide binding groove of the HLA molecule alters the repertoire of peptides presented by the HLA to the TCR to initiate the immune response/hypersensitivity.

1.3.1 Hapten Hypothesis

By definition haptens are drugs of molecular weight less than 1000D and as such are very small to induce an immune response on their own (Pichler 2008). However, when hapten molecules are attached to a larger molecule like proteins they can act as antigens that might elicit a pathogenic immune response. The hapten hypothesis is considered by some as the most plausible explanation of hypersensitivity reactions occurring due to the administration of drugs. When a hapten or its metabolite covalently binds to a protein (haptenization) it may cause cell stress leading to the secretion of cytokines. (Pichler, 2008).

The haptenated protein is recognised and taken up by professional antigen presenting cells (e.g., Langerhans cells in skin). The main function of these cells is to screen the cutaneous atmosphere for the presence of antigenic stimuli (Gorbachev and Fairchild, 2001). Antigen

loaded antigen presenting cells migrate to local lymph nodes, where they prime naïve T cells with drug-modified peptides derived from enzymatic antigen processing (Kalish, 1995). Fragments of drug-modified peptides are presented on major histocompatibility complex (MHC) molecules to naïve T cells (Fig 1.5). Clonal T-cell expansion lead to a population of antigen-specific T-cells that migrate to skin following the repeat exposure to target antigen. Secretion of cytokines and chemokines from inflamed skin and activated T- cells control the nature of the cellular immune response and the extent of tissue damage (Sebastiani et al., 2002).

Variation in balance between bioactivating and detoxifying pathways which leads to increased covalent adduct (hapten) formation is thought to be an important factor determining susceptibility to hypersensitivity. However, genetic polymorphisms in enzyme expression have been shown not to have a major impact on susceptibility (Umamaheswaran et al. 2014). Drug-protein-adduct can act as immunogen, with the potential to induce immune responses against drug-modified or native proteins. At present, this “hapten hypothesis” is the common model used by most groups exploring drug hypersensitivity reactions including halothane hepatitis (Bourdi et al., 1996) tienilic acid hepatitis (Lecoeur et al., 1996)¹, dihydralazine hepatitis (Bourdi et al., 1994) diclofenac hepatotoxicity (Hargus et al., 1994) β -lactam hypersensitivity, sulfonamide hypersensitivity (Shear et al., 1986), and certain forms antiepileptic drug hypersensitivity syndrome (AHS)(Shear and Spielberg, 1988, Pirmohamed et al., 1991, Leeder et al., 1992).

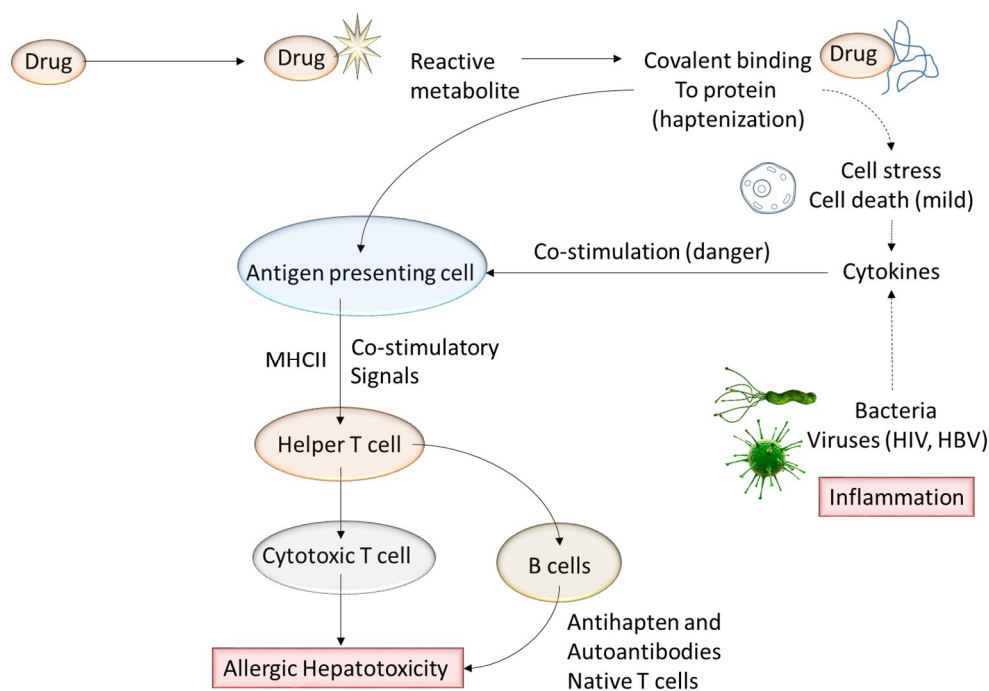


Figure 1.5 The general mechanistic pathway by which the hapten hypothesis is theorised to operate. Drug metabolism leads to covalent modification of protein. Protein are processed inside the cell, cleaved into peptide fragments and linked with MHC molecules then displayed to the cell surface. The T-cell receptor will then recognise the MHC/peptide complex. Antigens generated externally, attach to MHC class II molecules and stimulate a CD4⁺ T-cell response. Antigens generated inside the cell are processed through a proteasomal pathway, attach to MHC class I molecules, and stimulate a CD8⁺ T-cell response. According to the inflammatory stress hypothesis, IDILI-associated drugs interact with an otherwise benign episode of inflammation to precipitate liver injury.

1.3.2 Pharmacological Interaction of Drugs with Immune Receptors (PI Concept)

Beta-lactams need protein processing before antigen presentation, but other drugs have been found to directly bind to MHC and produce a T-cell response. Recent research suggests that drugs produce immune reactions by direct and reversible binding to MHC T-cell receptors. This theory is named the P-I concept. According to Adam (2011) the P-I concept states that “a drug is able to stimulate T cells directly without forming a hapten, in a HLA-dependent manner.” The concept was instrumental in explaining how some drug produce delayed hypersensitivity within a few hours of exposure. Drugs which have been proven to produce

this kind of specific labile binding are lidocaine, sulfamethoxazole, lamotrigine and carbamazepine (Posadas& Pichler 2007). In addition, glutaraldehyde-fixed cells which don't process proteins still allow for T-cell activation by specific drugs. The kinetics of T-cell drug-specific triggering are also too rapid to allow for antigen processing as a mechanism activation (Schnyder et al 1997; Hashizume et al 2002; Wu et al 2006; Keller et al 2010). The P-I concept describes a way in which drugs lacking hapten characteristics can bind directly with the antigen presenting cells and stimulate T-cells (Pichler 2008) (Figure 1.4).

1.3.3 Altered Self-Peptide Repertoire Model

In 2002, Mallal and his colleagues were the first group to describe the correlation between abacavir hypersensitivity syndrome and HLA-B*57:01(Mallal et al. 2002). The authors found that HLA-B*57:01 was present in 14 (78%) of the 18 patients with abacavir hypersensitivity, and in 4 (2%) of the 167 were abacavir tolerant patients. Subsequently, abacavir hypersensitivity was found only in patients expressing HLA-B*57:01, suggesting that the drug binds with specificity to the HLA-B*57:01 locus. This binding changes the chemical and stereochemical shapes of the antigen-binding cleft, which automatically alters the range of self-peptides that can bind to the groove. Thus 'immunological self' is altered, leading to CD8+ cell activation and autoimmune reactions, which present as the abacavir hypersensitivity reaction (Illing et al., 2012; Norcross et al., 2012; Ostrov et al., 2012). These experiments defined the “altered self-peptide repertoire model” of abacavir hypersensitivity.

1.3.4 Danger Hypothesis

The danger hypothesis proposed by Matzinger in 1994 suggests that pathogens and damage associated molecular patterns (DAMPs) play a critical role in immune activation (Matzinger, 2002). The immune system is more concerned with potential danger than foreignness (Gruchalla, 2001). Consequently an exogenous pathogen or chemical, or an endogenous

intracellular molecule secreted from necrotic cells, could not stimulate an immune response unless the immune system detects ‘danger’ (Matzinger, 1994, Pirmohamed et al., 2002, Li and Uetrecht, 2010). In the absence of danger, tolerance will result. Therefore, it is hypothesized that exposure to signals such as chemicals, drugs or infectious agents can trigger or amplify the innate immune response, with potential to activate the adaptive immune system. There are three signals essential for adaptive immune activation, signal one is also called the antigenic signal, and antigenic dererminants are presented to TCR in an MHC restricted manner. Signal two or co-stimulation occurs via the interaction between CD28 on the T cell and CD80 and CD86 molecules on the DC. Signal three derives from cytokines secreted by DC and are responsible for the differentiation and polarization of T-cell differentiation into various effector phenotypes such as Th1 and Th2. Fig 1.6 illustrates danger hypothesis.

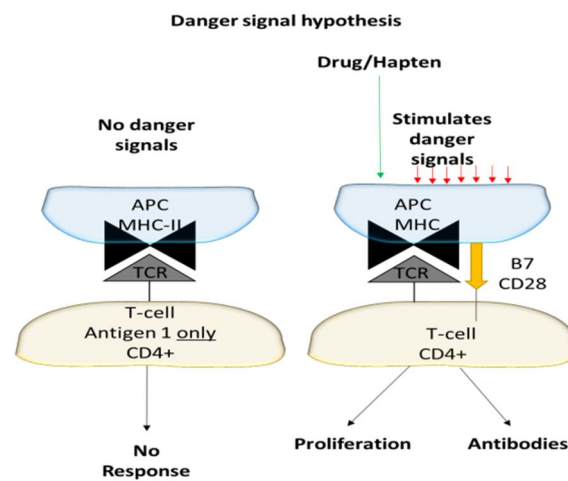


Figure 1.6: Danger signaling and drug hypersensitivity. Signals may come from chemicals exposure, physical trauma and /or infections. In drug-induced hypersensitivity syndrome, danger signalling lead to secretion of polarising cytokines that result in either Th1, Th2, Th17 or Th22 immune responses. Chemically reactive drug metabolites lead to an antigen (signal 1) in presence co-stimulatory B7 and CD28 to induce cell damage resulting in the generation of signal 2 and 3 required for an immune response.

1.4 Xenobiotic Drug Metabolism

In our daily lives, humans are exposed to a variety of substances which are foreign (xenobiotic). Such substance enter our body through air, foods and water. We are exposed to a variety of chemicals such as drugs, cosmetics, flavouring agents, food additives, detergents and so on. Our body handles all these xenobiotic substances entering our body in order to make them less toxic and rapidly excretes them to minimise damage. (Lamb et al 2009).

Xenobiotic substances may interact and modify body's metabolic functions and thereby become toxic. Several xenobiotic substances can interact with pharmaceutical agents and their metabolites and can cause toxic manifestations leading to ADRs. Some plausible mechanisms by which xenobiotic substances cause ADRs are: 1) interference with drug metabolism leading to generation of toxic substances capable of exerting an immune response; 2) interaction with the drug or its metabolites to produce toxic substances; 3) interference with drug metabolism and building up of drug metabolite in the blood to toxic levels leading to damage to organs involved in drug metabolism thus affecting the efficacy and safety of the drug. (Patterson et al 2010).

1.4.1 Drug Metabolism Enzymes

Drug metabolizing enzymes have a critical role in the processing of xenobiotics, drugs and endogenous substances. The biotransformation process carried out by drug metabolizing enzymes can be divided into two separate groups: phase I and phase II processes. By phase I process or reactions, parent compounds are transformed into more polar compounds through reactions such as N- and O- dealkylation, hydroxylation, N- and S-oxidation and deamination. The principal enzymes involved are cytochrome P450 (CYP). Phase II biotransformation also act on endogenous compounds and xenobiotics to form metabolites which can be easily excreted out of the body. Phase II transformations are normally conjugating reactions to

produce compounds which are more hydrophilic than the parent compound. A number of transferases enzymes are involved in phase II transformations (Jancova et al 2010).

1.4.2 Cytochrome P450

Cytochrome P450 (CYP) are a superfamily of proteins characterized by the presence of the heme cofactor. They use both small and large molecules as their substrates, and are classified into several groups including microsomal, mitochondrial and bacterial forms (Guengerich 2008). Cytochrome P450 (CYP) enzymes catalyses oxidative biotransformation of diverse type of xenobiotics including drugs. The nomenclature of Cytochrome P450 (CYP) enzymes is dependent on amino acid sequence similarity. A particular nomenclature has been developed by committee (figure 1.7). CYP1 (1A & 1B), CYP2 (2A, 2B, 2C, 2D & 2E) and CYP3 (3A) constitute the major CYP families with eight sub-families all together that account for the metabolic elimination of most drugs and xenobiotics in humans (Williams et al. 2004).

Inhibition of P450 can be the most common mechanism that can cause drug-drug interaction (DDIs) (Kalgutkar, Obach & Maurer 2007). P450 inhibition occurs via two means: reversible (competitive/non-competitive) or irreversible (mechanism-based inactivation). Inactivation involving the irreversible mechanism starts with bioactivation of xenobiotic to a reactive intermediate. Irreversible inhibition can also lead to undesirable drug-drug-interactions. An understanding of Cytochrome P450 (CYP) inactivation by drug candidate can help in rational drug design to circumvent P450 inactivation. This strategy along with pharmacokinetic data in humans can help to identify right molecules for drug development.

Kalgutkar, Obach & Maurer (2007) have analysed the biochemical basis of and structure-activity relationships of Cytochrome P450 (CYP) inactivation by xenobiotics. The magnitude of drug-drug-interactions can be predicted by using *in vitro* Cytochrome P450 (CYP) inactivation data for developing safer drugs. Pharmaceutical companies apply screening

paradigms to assess mechanism-based inactivation of Cytochrome P450 (CYP) along with human pharmacokinetic of the candidate drug to identify safer molecules.

Cytochrome P450 (CYP) is responsible for 75% of body's total metabolic reactions and is the main enzyme group involved in drug metabolism. While most drugs will be deactivated by Cytochrome P450 (CYP) directly or by facilitation of excretory process, some substances are activated to achieve their active state (Lamb et al 2009).

Cytochrome P450 (CYP) activity can be increased by inducing their biosynthesis or decreased by inhibition of its enzymes involved in biotransformation processes. Cytochrome P450 (CYP) is the main class of enzyme associated with ADRs. Any change in the activity of Cytochrome P450 (CYP) will affect the metabolism and clearance of drugs in the body. If a drug inhibits the Cytochrome P450 (CYP) mediated transformation and clearance of another drug, the other drug will accumulate in the body and build up toxic levels which can cause an ADR (Lamb et al 2009).

1.4.3 Flavin-Containing Monooxygenases (FMOs)

FMOs are a family of enzymes that protect us from the threat of chemical substances present in our environment. They catalyse the oxidation of foreign chemicals such as pesticides, therapeutic agents, dietary food substances and other substances present in the environment. FMOs are not as exhaustively studied as cytochrome P450. Recent studies have led to greater appreciation of the role played by FMOs in dealing with pharmacological and toxicological effects of substances on human body. Unlike CYPs, FMOs are not induced or inhibited by foreign substances which give them advantage over CYPs in being less prone to drug-drug interactions. According to Philips and Shephard (2008) the differences in FMOs activity between individuals are greatly due to inheritance and not due to the environmental factors.

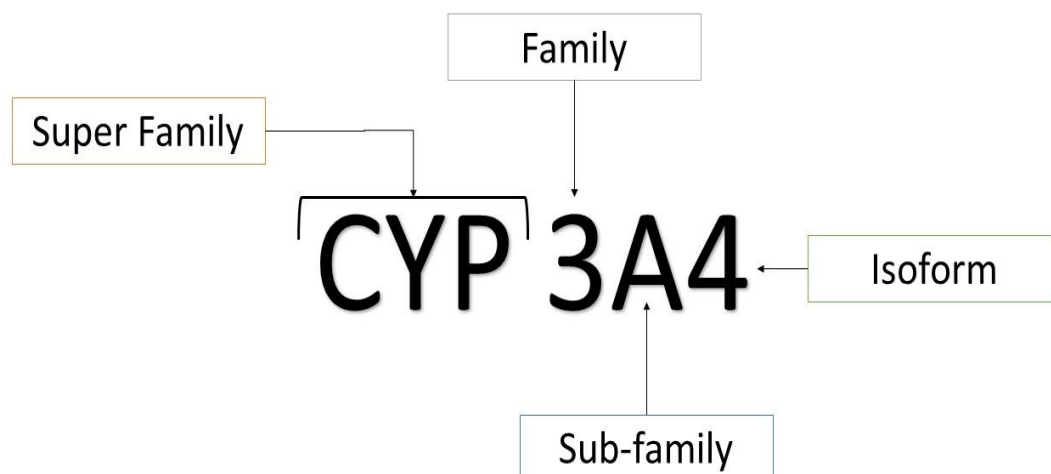


Figure 1.7: Nomenclature of cytochrome P450.

1.4.4 Peroxidases

Peroxidases are critical in inflammation and exacerbation of diseases like asthmatic disorders. When inflammatory cells like neutrophils and eosinophils are activated during inflammatory conditions their secretory products are associated with the clinical progression of the disease. When activated, neutrophils and eosinophils secrete unique peroxidases. According to Reszka et al (2011), these unique cell peroxidases are functionally similar to lactoperoxidase present in the lining of the lung (Reszka et al, 2011). Peroxidase enzymes catalyse the production of reactive drug metabolites in immune cells (Besser et al. 2009). The most common examples of peroxidases involved drug metabolism are Myeloperoxidase (MPO), eosinophil peroxidase (EPO), and lactoperoxidase (LPO) (Tafazoli and O'Brien 2005).

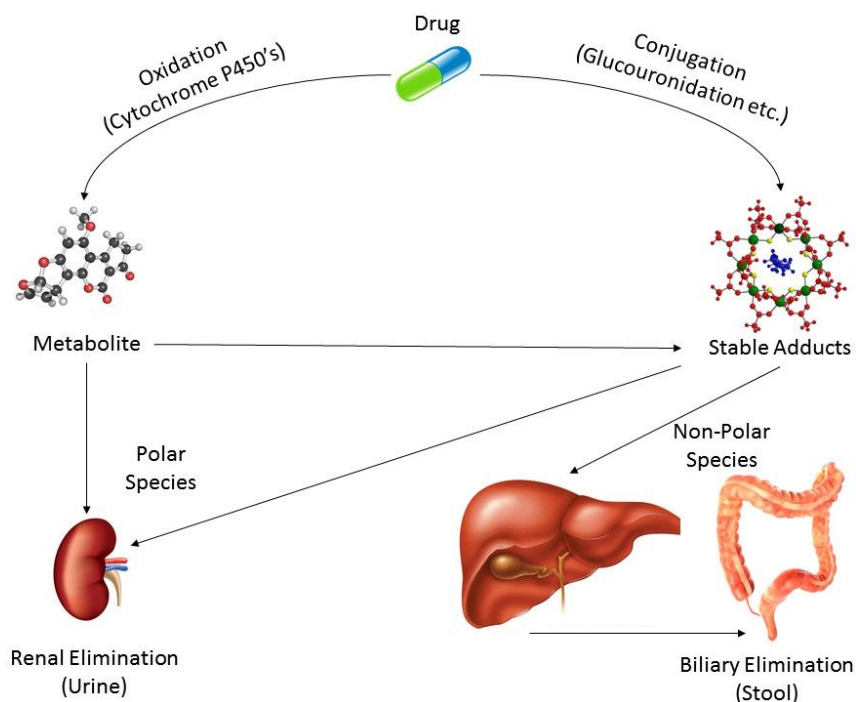


Figure 1.8: A general overview of the drug metabolism pathways observed within the human body.

1.5 Phase I Drug Metabolism: The Modification Stage

Phase I of drug metabolism is the modification step during which certain groups are added to drug molecule to facilitate conjugation during phase II. The phase I reactions are of three types: oxidation, reduction and hydrolysis. Following these steps, there can be several situations with respect to the new compound formed: it can have same activity, it can have a different activity, it can be safer or less toxic or can be more toxic. If the reactions involve an increase in water solubility, according to Schonborn (2010), the new compound formed can be rapidly excreted at this stage.

- (i) **Oxidation:** During oxidation the drug molecule undergoes insertion of an oxygen atom to form unstable intermediate. The intermediate rearranges into the product of drug metabolism. These reactions are catalysed by cytochrome P450. The reactions that take

place include hydroxylation, epoxidation, dealkylation and deamination and dehalogenation. Example of drugs that undergo oxidation are codeine, omeprazole and paracetamol (Schonborn (2010)).

- (ii) Reduction: These reactions are also catalysed by cytochrome P450 and often are performed under anaerobic conditions. Several drugs are known to undergo reduction. For example, prednisone (a prodrug) undergoes reduction into active glucocorticoid prednisolone and warfarin is inactivated by transformation of a ketone group (Schonborn (2010)).
- (iii) Hydrolysis: This step is catalysed by esterases and amidases. Some drugs which undergo this step are prilocaine which undergoes hydrolysis by amidases in liver and remifentanil undergoes plasma ester hydrolysis resulting in a short elimination half-life (Schonborn (2010)).

1.5.1 Phase II Drug Metabolism: The Conjugation Stage

This stage increases the drugs solubility by the addition of a conjugating agent. This process facilitates excretion of the drug in bile and urine. Most phase II reactions inactivate drugs or the active molecule formed during the phase I reactions. Some important phase II reactions are:

- (i) Glucuronidation: This is the major route of propofol metabolism for allowing excretion through liver and kidneys. By glucuronidation, morphine is metabolized to morphine - 6-glucuronide which is 13 times more potent than the parent compound. According to Schonborn (2010), the midazolam also undergoes this reaction to form a metabolite having prolonged sedative effect. UDP-glucuronosyltransferases (UGTs): These are a superfamily of enzymes that perform the detoxification pathway for chemicals, dietary

components, carcinogenic agents and their oxidized metabolites. According to Wells et al (2004), 40-70% of clinical drugs undergo glucuronidation. Mackenzie et al (2008) point out that UGTs are critical for the incorporation of UDP-hexose to nucleophilic atom in the acceptor molecules. Jancova and Siller (2012) reported that the active site of UGTs is localized in the luminal space of the surface of endoplasmic reticulum (ER) (Mackenzie et al, 2008).

- (ii) Sulphation: Among drugs which undergo this reaction is paracetamol. Paracetamol (40%) also undergoes N-hydroxylation to N-acetyl-p-aminobenzoquinonimine (NAPQI) which is toxic. This is then susceptible to sulphation prior to elimination.
- (iii) N-Acetylation: The various forms of the enzyme give rise to variable rates of acetylation in different individuals. Individuals are divided into slow or fast acetylators and the effects administration of such as isoniazid and hydralazine. (Barranco, Minor & Solomon 1976)

1.6 Dapsone

Dapsone was synthesized in 1908 and continues to be used as a powerful therapeutic agent for skin diseases (Wozel, 1989). Dapsone has an antibacterial spectrum and is the drug of choice for leprosy since the 1950s. It can also be used for a broad spectrum of ailments: acne (Prendville, Logan & Russel-Jones 1988), bullous pemphigoid as an adjunct (Jeffes & Ahmed 1989), pneumonia caused by *Pneumocystis carinii* (Leoung, Milles & Hopewell 1986), dermatitis herpetiformis (Leonard 1991), relapsing polychondritis (Barranco, Minor & Solomon 1976), vesico bullous lesions of lupus erythematus as first line therapy (Hall et al 1982), vasculitis syndrome (Forston et al, 1986), spider bite (Bennavides & Moncada 1990), pyoderma gangrenosum (Brunsting & Goeckerman, 1930) and pemphigus herpetiformis (Leonard 1991). Dapsone is absorbed well from gut and its metabolism is directed through *N*-

acetylation and *N-hydroxylation* (oxidation). People differ in the rate of acetylation resulting in slow and fast acetylators in the patient population. ADRs due to dapsone normally develop after six weeks of initiation of the drug (Rege, Shukla & Mascarenhan 1994). Kannan et al (2009) found dapsone effective both in leprosy and non-leprosy patients causing no serious hematological complications; they did not find any case of dapsone hypersensitivity syndrome. Kannan et al (2009) have reported peripheral neuropathy as the main ADR of dapsone users in 35% patients of leprosy patients and 18% of non-leprosy patients. Dapsone hypersensitivity differs from other drug reactions because its occurrence takes place after prolonged exposure to the drug and the reactions can occur even after six months or more of exposure. Dapsone hypersensitivity syndrome have a complex mix of clinical manifestations, such as eosinophilia, jaundice, fever, and cutaneous manifestations like erythroderma, maculopapular eruption, epidermal necrosis and Stevens-Johnson syndrome (Richardus & Smith 1989; Smith 1988) as well as internal organ damage. The hematologic adverse effects associated with dapsone are methemoglobinemia and hemolytic anemia which are believed to be dose-dependent and caused by the dapsone hydroxylamine metabolites, which is potent oxidants. Though dapsone is excreted by kidneys, it has significant enterohepatic circulation.

Severe cases of ADR caused by dapsone involve hypersensitivity syndrome (DHS) which is potentially fatal. Dapsone hypersensitivity syndrome involves irreversible organ damage, which can be treated by timely use of steroids in oral formulation (Vinod 2013). The hypersensitivity reported by dapsone occurs about 20 days after its administration and symptoms persist even ten days after its discontinuance. Dapsone hypersensitivity can develop even several weeks to six months after drug intake. In rare cases dapsone hypersensitivity is also reported to cause bone marrow suppression and pancytopenia in addition to fever, rash, and hepatosplenomegaly (Vinod 2013).

Chapter 1

During the long intervening period of its elimination half-life of 24 to 30 hours, dapsone metabolites may be the cause for eliciting ADRs (Gennaro et al, 2000). The hydroxylamine metabolite is responsible for methemoglobinemia, hypersensitivity syndrome, neuropathy, and agranulocytosis (Kannan et al 2009).

Several drug-drug interactions (DDIs) have also been observed with dapsone. Interaction with the drug fluconazole reduces production of toxic metabolite and reduction of adverse reaction of dapsone, while cimetizine reduces methemoglobinemia due to dapsone (Kannan et al 2009).

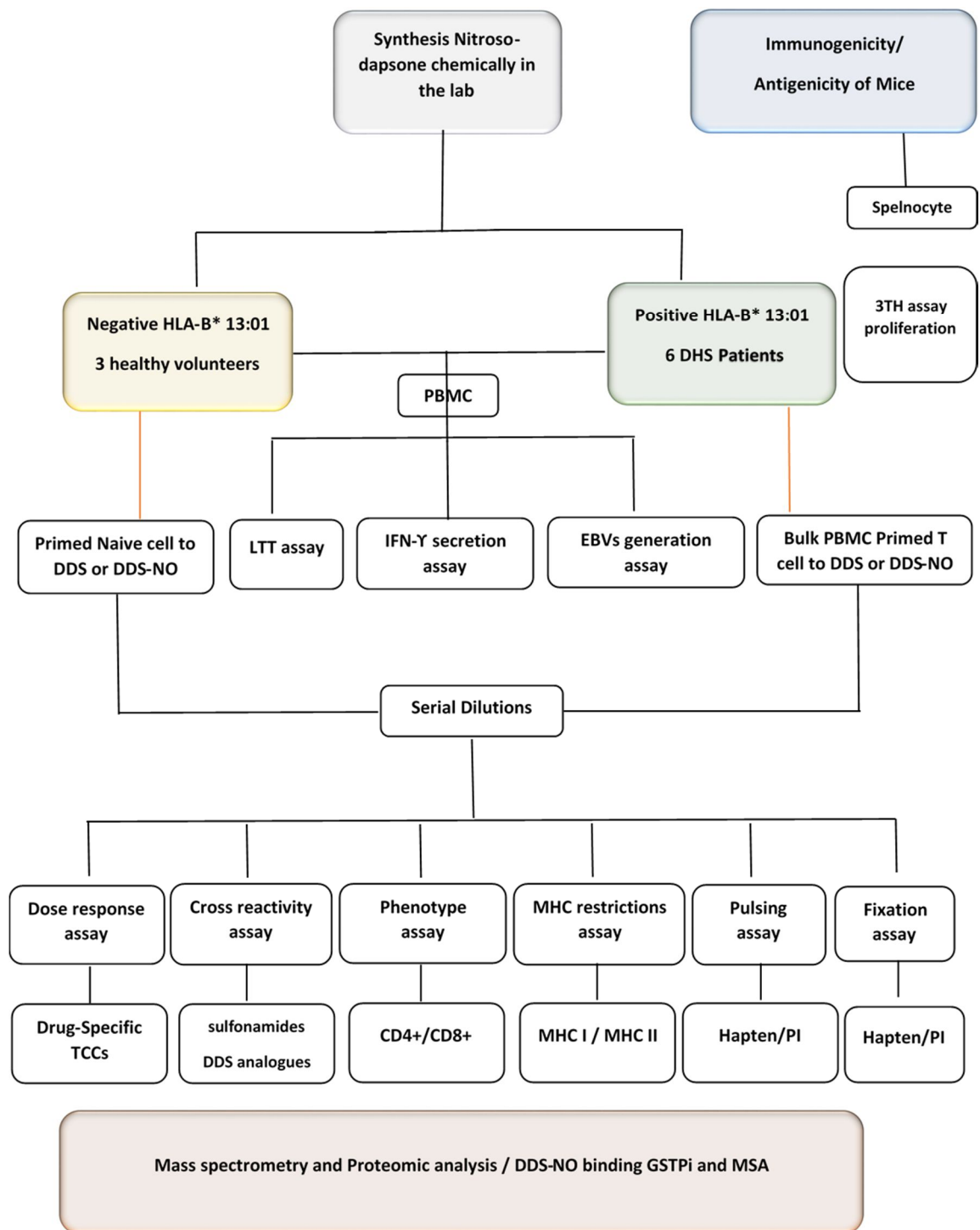


Figure 1.9: Flow chart illustrating the project design.

Chapter 1

In 2013, Zhang and his colleagues, considered that there is a relationship between HLA-B*13:01 and dapsone hypersensitivity syndrome especially among patients with leprosy disease (Zhang et al, 2013). Based on Zhang et al (2013) study we designed our project to describe the association between T-cell activation and dapsone hypersensitivity with positive or negative HLA-B*13:01 donors. Firstly, synthesis of Nitroso Dapsone (DDS-NO) the aims of this study is to explore the immunogenicity of the DDS and DDS-NO in human test systems or mouse system model. As summarised above see figure 1.9.

Chapter 2: Synthesis nitroso-dapsone and priming of naive T-cells to dapsone and nitroso dapsone.

2.1 Introduction:	55
2.1.1 Mechanism of Action of Sulfonamides:	57
2.1.2 Metabolism of sulfonamides:	58
2.1.3 Hypersensitivity of sulfonamides:	59
2.1.4 Chemical synthesis of sulfonamides metabolites:	59
2.1.5 Dapsone (4,4'-diaminodiphenylsulfone, DDS)	60
2.1.6 Absorption of Dapsone:	60
2.1.7 Distribution of Dapsone:	61
2.1.8 Dapsone metabolism	62
2.1.9 Dapsone and Hypersensitivity	62
2.1.10 Chemical synthesis of dapsone metabolites.	63
2.1.11 Dapsone Analogues	65
2.2 Aims Of The Investigation	69
2.3 Materials And Methods	70
2.3.1 Study Area and Approval	70
2.3.2 Chemicals and reagents	70
2.3.3 Culture medium	71
2.3.4 Isolation of peripheral blood mononuclear cells (PBMC)	71
2.3.5 Isolation of PMBC and separation of subsets of T-cells	71
2.3.6 Generation of dendritic cells (DCs)	72
2.3.7 Lymphocyte Transformation Test (LTT)	72
2.3.8 Priming of naïve T cells to DDS and DDS-NO	72
2.3.9 [³ H]-Thymidine incorporation proliferation and IFN- γ secretion assays	73
2.3.10 ELISpot Assay	73
2.3.11 Synthesis of Nitroso-Dapsone	74
2.3.12 Mass spectrometric analysis of DDS-NO binding to glutathione	74
2.3.13 Luminex assay	75
2.3.14 Sample preparation	75
2.4 Results Of The Investigations	76
2.4.1 Synthesis and characterization of DDS-NO	76
2.4.2 Toxicity assay	79
2.4.3 Lymphocyte transformation test (LTT)	79
2.4.4 Priming of naïve T-cells from healthy donors to DDS and DDS-NO	80
2.4.5 Cytokine production by DC cultured with DDS, SMX, DDS-NO or SMX-NO	82
2.5 Discussion	83

2.1 Introduction:

Sulfonamides are the base of various drugs and the pioneer chemotherapeutic antibacterial agents used systematically in modern medicine (Hansch et al., 1990). They have been in routine medicine practice for 70 years and are most widely used throughout the world against numerous bacterial infections and other diseases (Ozbek et al., 2007). From anti-bacterial activity to anti-fungal (Isik et al., 2009), and from antineoplastic (Tiwari et al., 2006) to antiviral (Lu et al., 2007), sulfonamides have revolutionized medicinal chemistry since their discovery. It has also been reported that they are important in the treatment of rheumatoid arthritis (Levin et al., 2002) and Alzheimer's disease (Roush et al., 1998). Literature shows that sulfonamides are an integral component of many anticonvulsant, antihypertensive, antipsychotic, diuretic, and hypoglycemic drugs (Shah et al., 2013). Other than these uses, sulfonamides also show the ability to inhibit the activity of various enzymes (Supuran et al., 2003). Numerous studies have stated that a considerable number of highly prescribed drugs have a sulfonamide subunit. According to Ding et al. (2013) and Graul et al. (2013) 10% of the top 100 drugs recommended in 2011-2012 had a sulfonamide moiety.

Sulfonamides were first discovered by Domagk et al. in 1935 while they were studying Prontosil 1 metabolism. Prontosil is metabolized into an active sulfonamide agent in vivo which then interferes with the bacterial growth (Figure 2.1) (Patrick, 2009).

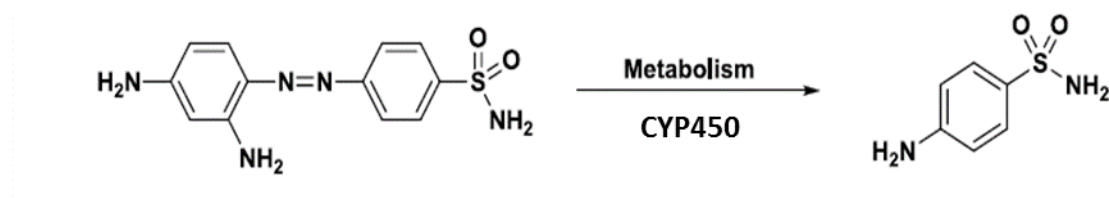


Figure 2.1- Metabolism of prontosil.

Sulfonamides have SO₂-NH₂ functional group in their structure (Warshakoon et al., 2009). Their general formula is RSO₂NH₂ where R represents any organic group. Sulfonamides differ

in the N1 position which alters the strength, solubility and pharmacokinetic properties of the molecule (Genc et al., 2008). Based on chemical structure there are two different groups of sulfonamides. The first group is sulfonylarylamines in which sulfonamide moiety is directly attached to a benzene ring and there is also an amine ($-\text{NH}_2$) moiety at the N4 position. Non-sulfonylarylamines are the second group in which there is also a sulfonamide group attached to benzene ring but there is no amine moiety attached at the N4 position (Johnson et al., 2005). The fact that some sulfonamides act as antibiotics while others are non-microbial agents is due to the difference in the R components and other variable groups Figure 2.2;

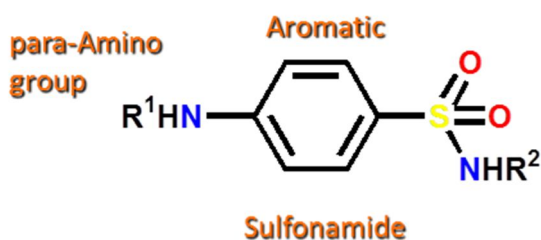


Figure 2.2- Structural formula of sulfonamides

The para-amine group (R^1HN), aromatic ring and sulfonamide groups are essential groups of the sulfonamide drug. R^2 can vary in different sulfonamides (Vree. Et al. 1986).

The pharmacokinetics of sulfonamides differ based on lipophilicity and hydrophilicity due to differences in the nitrogen containing Para-amino and sulfonamide group. The more lipid soluble the drug is, the more it becomes bound to proteins in the plasma (Fujita et al. 1867). The P-amine group in most of the sulfonamides is a very important part of the drug because any change and modification in the Para-Amino group results in the loss of activity of the drug (Anand N et al. 1996). The length of the amide group determines the antibacterial activity of the sulfonamide drug. The antimicrobial activity of the drug increases with increase in the length of the alkyl group. Thus, sulfamethoxazole has a long aliphatic hydrocarbon group and therefore has wide spectrum antibacterial activity (Ozbek et al., 2007).

It should be noted that amide groups cannot be ionized and this lowers the polarity of sulfonamides group. The alkyl group increases the hydrophobic nature of the drug thus, making it easily to absorb and cross the gut wall. Inside the gut wall cells, the drug is metabolized by peptidases that generate primary amines. Primary amines are ionizable and thus easily interact to perform their action. (Maren et al., 1976). Dapsone as a sulfonamide acts in several different ways as it is used to treat a variety of illnesses and diseases. It has an anti-bacterial effect by inhibiting bacterial growth, which is especially important when used to treat leprosy. But it is also used for the treatment of skin conditions that are not caused by bacteria. Dapsone shows that it can be used as an anti-inflammatory and as a therapy in auto immune diseases (Qiang, 2013).

2.1.1 Mechanism of Action of Sulfonamides:

Sulfonamides are bacteriostatic agents and are competitive enzyme inhibitors. They mimic the structure of the substrate of PABA, binding to their active site, thus blocking access to PABA in a reversible manner Figure 2.3. (Seydel et al., 1968).

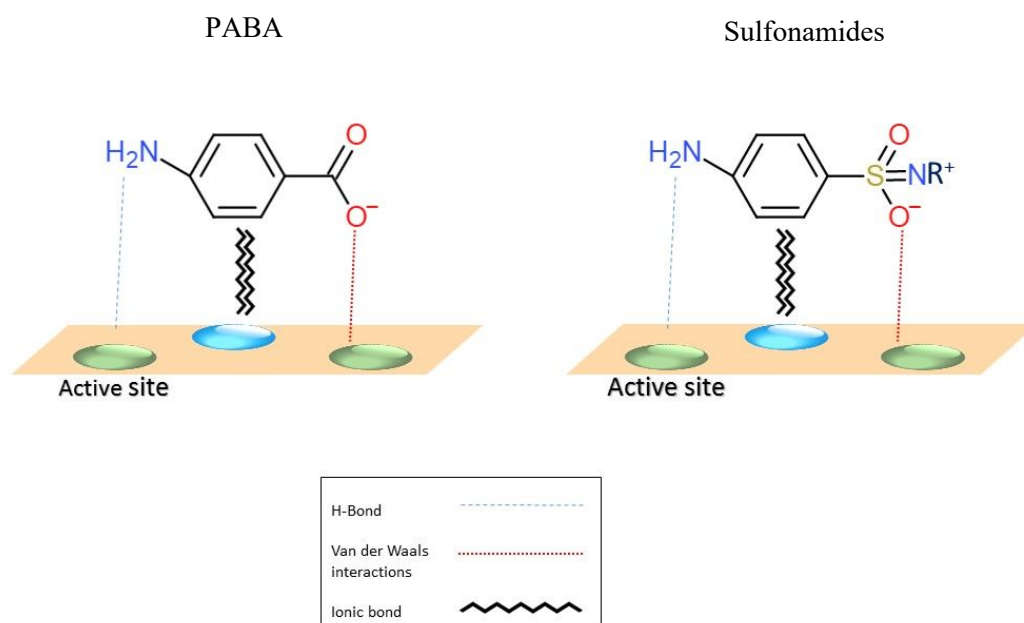


Figure 2.3- Mechanism of action of sulfonamides.

The antibacterial activity of sulfonamides is due to their resemblance to p-amino benzoic acid (PABA) which is required by bacteria for synthesis of DNA. The basic mechanism involves the competition of sulfonamides with PABA for the enzyme dihydropteroate synthetase, resulting in the inability of bacteria to form dihydrofolate which is essential for DNA synthesis of bacteria (Caddick, 2005).

2.1.2 Metabolism of sulfonamides:

Most of the sulfonamides are detoxified by the liver and are metabolized by acetylation. The acetylation decreases the hydrophobicity of the drug thus rendering it inactive. Sulfonamide also form hydroxylamine intermediate which are implicated in hypersensitivity reactions (Lipmann & Tuttle 1945). These are discussed in more details below.

2.1.3 Hypersensitivity of sulfonamides:

Sulfonamide therapy has been associated with hypersensitivity reactions including skin eruptions, and fever (Rieder et al., 1989). The exact mechanism of these hypersensitivity reactions is complex and not well understood. Various metabolic and genetic factors contribute to individual susceptibility. It has been hypothesized that these reactions occur because of the formation of reactive derivatives (Svensson, 2003). Many studies have shown that these metabolites are produced *in-vivo* by the cytochrome P450 (CYP) monooxygenase system (Cribb and Spielberg, 1992), mainly CYP 2C9 (Cribb et al., 1995). 'Sulfa Allergy' is the term used for all the adverse effects of sulfonamide drugs. Hypersensitivity reactions are reported in 3-4% of the patients receiving sulfonamides drugs (Kucera et al., 1996). However, this increases in disease states such as cystic fibrosis and HIV due to immune deficiency state of patient. The signs and symptoms include anaphylactic shock, skin eruption and reactions targeting internal organ (Knowles et al., 2001). Many Antibiotics such as sulfonamides,

penicillins, macrolides and clindamycin are involved in acute generalised exanthematous pustulosis. It is possible that rashes which start off as maculopapular eruptions may extend into full-scale toxic epidermal necrolysis or the Steven-Johnson syndrome. (Schmid et al. 2002; Sidoroff et al. 2007).

2.1.4 Chemical synthesis of sulfonamides and their metabolites

Because of their importance in the pharmaceutical industry and appearance of drug-resistant strains in clinical applications (Cassell and Mekalanos, 2001), a number of chemical methods have been reported for the synthesis of new sulfonamide derivatives (Moreno-Diaz et al., 2008 ; Dominguez et al., 2005 ; Ozbek et al., 2007). The most common method for the synthesis of sulfonamides is sulfonylation of amines with chlorides in the presence of a base (Humljan and Gobec, 2005). Other methods which have been reported include but are not limited to synthesis using thiols (Wright and Hallstrom, 2006), from sulfonic acid (De Luca and Giacomelli, 2008), using sulfonamides (Revankar et al., 1990) and transition metal catalyst (Shekhar et al., 2011). A novel method of sulfonamides production is production from sulfenamides such as 2-amin-9H-purine-6 sulfenamide with the help m-chloroperoxybenzoic acid (m-CBPA) which slightly increases the yield of sulfonamide production from 49% to 53%. (Revankar et al., 1990).

2.1.5 Dapsone (4,4'-diaminodiphenylsulfone, DDS)

Dapsone is the main drug used for the treatment of leprosy (Figure 2.4)(Makarov et al., 2006). It is an analogue of sulphone and is the simplest of all in the group (Zhu and Stiller, 2001). Dapsone does not contain the exact complete sulfonamide moiety although it contains a N4 arylamine like the sulfonylarylamines of the sulfonamides (Brackett et al., 2004). The mechanism of action of dapsone against bacteria is same as of sulfonamides; they also compete with PABA and inhibit the synthesis of dihydrofolic acid (Coleman, 1993).

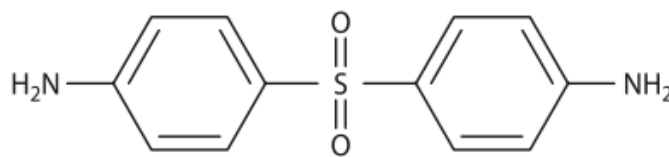


Figure 2.4-Structural formula of Dapsone.

2.1.6 Absorption of Dapsone:

Dapsone is well absorbed from the gut after its oral administration with a bioavailability of more than 80%. With a single dose, the peak level of the drug is obtained after 2-6 hours. A steady state level is obtained after continuous therapy of 10 days. The serum drug concentration does not change unless the dosage of the drug is changed. The half-life of dapsone is about 15-30 hours (Katz 1999, Lang 1979, Zhu and Stiller 2001, Zuidema et al. 1986).

In addition to its antileprosy activity, dapsone has shown effectiveness in patients with rheumatoid arthritis (Swinson et al., 1981), systemic lupus erythematosus (SLE) (Hall et al., 1982), and thrombocytopenia (Godeau et al., 1993). Studies have shown that many derivatives of dapsone have been synthesized and they also have antibacterial and anti inflammatory properties (Kansyl et al., 1992).

2.1.7 Distribution of Dapsone:

Dapsone is 70-90% bound to plasma proteins. The metabolite of dapsone; monoacetyldapsone MADDS, is 100% bound to plasma proteins. Dapsone is almost equally distributed to all body tissues including skin, liver, renal system, heart and the intestines. Dapsone can cross the blood brain barrier and pass into the brain. Data also shows that dapsone can cross the placenta resulting in cases of neonatal hemolysis, documented after oral administration of dapsone to pregnant women (Katz 1999, Lang 1979, Zhu and Stiller 2001, Zuidema et al. 1986).

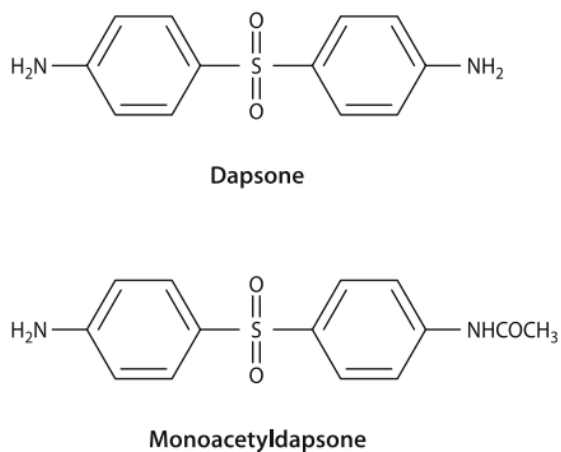


Figure 2.5 Acetylation of Dapsone.

2.1.8 Dapsone metabolism

Metabolism of dapsone in humans involves N-acetylation producing monoacetyldapsone (MACDDS) (Gelber et al., 1971) (Figure 2.5). Dapsone also undergoes hydroxylation by cytochrome P450 isozymes CYP2C19, CYP2C9, CYP3A4 and CYP2E1 to produce dapsonehydroxylamine (DDS-NOH) (Ganesan et al., 2010). The oxidation of dapsone hydroxylamine then produces Nitroso metabolite called nitroso-dapsone Figure 2.6 (Coleman, 1995).

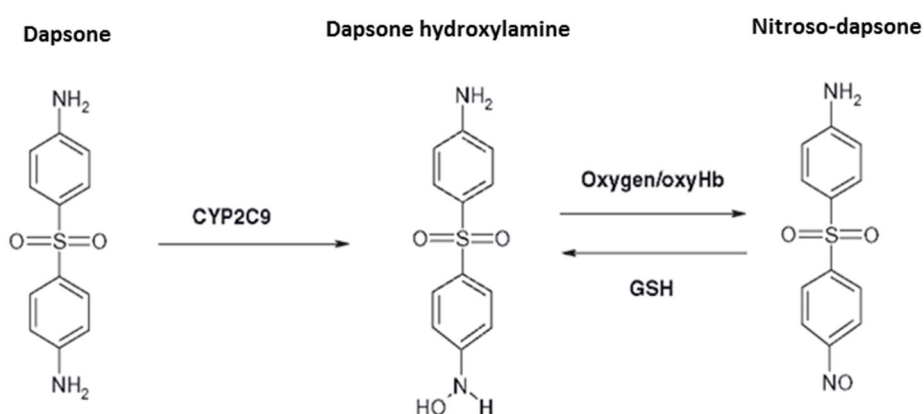


Figure 2.6 Metabolic oxidation of dapsone: DDS is converted to DDS-NOH by CYP2C9. Dapsone hydroxylamine is then autoxidized in DDS-NO.

2.1.9 Dapsone and Hypersensitivity

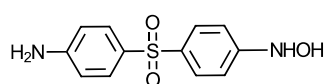
The use of dapsone is also associated with many adverse effects including pharmacological reactions such as anemia, hemolysis, methemoglobinemia, and allergic reactions such as dapsone hypersensitivity syndrome (Cucinell et al., 1972; Coleman et al., 1996). The direct toxic effects of dapsone are caused by dapsone oxidative metabolites. It has been proposed that dapsone-induced hypersensitivity syndrome is immune-mediated and involves the activation of T-lymphocytes (Ben m'rad et al., 2009). However the nature of drug-antigen that stimulates T-cell has not been defined.

Lowe and his colleague were first to report dapsone-induced hypersensitivity syndromes (Lowe and Smith, 1949). Later Allady and Barnas gave the aforementioned term to syndrome and described the clinical features further (Allady and Barnas, 1951). Data suggest that multidrug therapy in leprosy patients has led to an increase in the incidence of dapsone hypersensitivity syndrome in the past decade. The syndrome is noted after a month of therapy with dapsone and signs and symptoms include fever, nausea, vomiting, malaise, deranged liver function tests, anemia, dermatitis, eosinophilia, leukocytosis and other related symptoms. The clinical picture and symptoms are quite similar to infectious mononucleosis disease however there is no evidence of the Epstein-Barr virus (EBVs) sensitivity or toxoplasmosis. (Prussick et al. 1996)

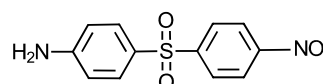
There is no guideline for the management of dapsone-induced hypersensitivity syndrome, however the convention is to stop the administration of dapsone and start introducing corticosteroids. Steroids are introduced and given for a long duration as dapsone has a long half-life in tissues due to enterohepatic circulation. The exact mechanism of the dapsone hypersensitivity syndrome is unknown however the response to steroids and the clinical picture of eosinophilia suggest that the syndrome is an idiosyncratic hypersensitivity reaction (Mofenson et al. 2009).

2.1.10 Chemical synthesis of dapsone metabolites.

Dapsone was first synthesized in 1908 by Fromm and Wittman (Fromm and Wittman, 1908). As a result of increase in drug resistance by bacteria, and their hypersensitive reactions, there have been efforts to synthesize the metabolites and derivatives of these drugs *in vitro* to understand the mechanism of toxicity and investigate the effects of these metabolites relative to dapsone drug (Kansyl et al., 1992). First experiments to produce metabolites of dapsone *in vitro* were conducted by Uetrecht et al (Uetrecht et al., 1998). Dapsone was incubated with polymorphonuclear cells (PMN)- and zymosan-activated human PMN. Gas chromatography and high-pressure liquid chromatography were used to characterize the hydroxylamine metabolite of dapsone. Further oxidation of dapsone hydroxylamine resulted in the generation of nitroso derivatives of dapsone (Drayer et al., 1974; Uetrecht et al. 1988). Despite these efforts, nitroso-dapsone was not generated in sufficient quantities for described *in-vitro* or *in-vivo* challenges of toxicity of immune reactions.



(A)



(B)

Figure 2.7 (A) Chemical structure of Dapsone hydroxylamine. (B) Chemical structure of Nitroso dapsone.

2.1.11 Dapsone Analogues

To overcome the common adverse effect of hemotoxicity following administration of dapsone, structural analogues have been synthesized that retain pharmacological activity, reduce toxicity and increase the therapeutic window of dapsone. It has been found that replacement of sulfone group of dapsone by sulfur, oxygen, methylene, or carbonyl substituents significantly reduce

the hemotoxicity in the human erythrocytes (Mahmud et al., 1997). There is a lot of data and experimental studies on the bioavailability and pharmacokinetics activity of dapsone and related dapsone analogues. Studies have shown that many analogues of dapsone have anti-inflammatory properties (Kansyl et al., 1992). It has been found that dapsone and its analogues are also useful for preventing and for treating pathologies involving memory loss such as Alzheimer's and related neurodegenerative disorders. However, although many analogues of dapsone have been synthesized for better pharmacological activity, there are only a few studies on the structure–toxicity relationships of these compounds (Coleman et al., 1991).

2.1.11.1 Dapsone analogue 3, 4' DDS

3, 4' DDS (3, 4'- diaminodiphenyl sulfone) is also an aniline derivative and belonging to the group of synthetic sulfones. Like DDS, its structure contains a sulphur atom linking to two carbon atoms. It also contains two free amino groups in the structure and thus can act as an antimicrobial agent (Figure 2.8, II). The main structural difference between DDS and 3,4' DDS analogue is the presence of an amino group in a different position. This small positional change provides greater pharmacological activity than dapsone. It is a water soluble compound and its absorption is complete and regular from the gastrointestinal tract. Although it has better pharmacological activity than dapsone, Coleman et al (1991) suggested that the 3,4' DDS produces significant amount of methaemoglobin in human erythrocytes.

2.1.11.2 Dapsone analogue 3DDS

3, 3' DDS (3, 3'- diaminodiphenyl sulfone) is also an aniline derivative belonging to the group of synthetic sulfones (Figure 2.8, III). It contains two free amino groups in the structure and thus can act as an antimicrobial agent. The only structural difference between DDS and 3, 3' DDS analogue is the presence of amino groups in different position. Furthermore, this small positional change of amino groups provides it greater pharmacological activity than DDS and

good pharmacokinetics properties.³ 3'DDS does not produce significant methaemoglobin in human erythrocytes and as a consequence, is associated with significantly less toxicity compared with DDS (Coleman et al., 1991). It has been shown that the haemotoxicity of DDS analogues is related to the electron-withdrawing nature of the 4-substituent (Mahmud et al., 1997).

2.1.11.3 Dapsone analogue MACDDS

It is also an active analogue of DDS and chemically known as mono-N-acetyl DDS (MACDDS). The main structural difference between MACDDS and DDS is the presence of an acetyl group (Figure 2.8, IV). Due to the presence of one free amino group in the structure, MACDDS can still act as an antimicrobial agent. It has ten times stronger plasma protein binding (97-100%) when compared with DDS (David et al., 2011). Although it possesses better pharmacokinetic properties than DDS, it is one of the most potent compound at inducing methaemoglobinemia (Coleman et al., 1991).

2.1.11.4 Dapsone analogue DACDDS

This dapsone analogue is also known as Acedapsone (4, 4'-diacetyl-diphenyl sulfone) and it doesn't contain a free amino group in the structure (Figure 2.8, V). The most important feature of DACDDS is that it does not produce a significant amount of methaemoglobin (Coleman et al., 1991). Thus, it is not able to produce significant hemotoxicity. Unlike all formulations of sulfones, which are taken orally, DACDDS would need to be administered intramuscularly. It releases the drug slowly from the administered site but, after release metabolism occurs rapidly and converts into the active form, DACDDS has a long plasma half-life thus, it possible to formulate sustained release injectable drug delivery strategies(David et al., 2011).

2.1.11.5 Dapsone analogue MACDDT

The dapsone analogue MACDDT is a thioaniline derivative chemically known as 4-acetyl-4-amino-diphenyl thioether (Figure 2.8, VII). The main feature of the molecule is a diphenyl ring with a sulphur atom linking to two carbon atoms. The main structural difference between MACDDT and DDS is the presence of a sulfide or thioether group instead of sulfone group and an additional acetyl group. Due to the presence of one free amino group in the structure, MACDDT can act as an active antimicrobial agent like DDS. It has been reported that MACDDT can cause significantly greater methaemoglobin formation than DDS (Coleman et al., 1991).

2.1.11.6 Dapsone analogue DACDDT

This DDS analogue is also a thioaniline derivative and its chemical name is 4, 4'-diacetyl-diphenyl thioether DACDDT (Figure 2.8, VIII). It doesn't contain a free amino group in the structure. Thus, this analogue is only active after the conversion to metabolites containing one or two free amino groups. It also forms a significant amount of methaemoglobin and as a consequence causes haemotoxicity (Coleman et al., 1991).

2.1.11.7 Dapsone analogue DDT

DDT is another thioaniline derivative of DDS and its chemical name is 4, 4'-diaminodiphenyl thioether (Figure 2.8, IX). Unlike DDS, it contains a sulfide group (a sulphur atom linking to two carbon atoms) instead of a sulfone group. It also contains two free amino groups in the structure and thus can act as an antimicrobial agent. The most important feature which makes it superior to 3, 4' DDS and 3, 3' DDS is that, it does not produce significant amounts of methaemoglobin (Coleman et al., 1991). Thus, it is not able to produce significant hemotoxicity in the human.

2.1.11.8 Dapsone analogue DDE

DDE is known as 4, 4'-diaminodiphenylether. It contains two amino groups in the structure and that is why it can act as an antimicrobial agent. DDE has a diaminodiphenylether group and this is the most exclusive structural feature which makes it superior than other DDS analogues (Figure 2.8, VI). Due to the absence of sulfone group it does not cause a significant amount of methaemoglobin (Coleman et al., 1991).

2.1.11.9 Dapsone analogue F8 DDS

F8 DDS or 4, 4'-diaminooctofluoro-diphenyl sulphone is a fluorine containing analogue of DDS. The main feature of which is a diphenyl ring with a sulphur atom linking to two carbon atoms with fluorine atom substitution (Figure 2.8, X). This compound also contains two free amino groups in the structure which makes it an active antimicrobial agent. It does not produce a significant amount of methaemoglobin in humans (Tingle et al., 1988). The decrease in toxicity observed with the electron-withdrawing octofluorodiphenyl groups may be an effect of the drug density on the amino group which would reduce the ability of cytochrome P450 to N-hydroxylate the compound (Yin et al., 1995).

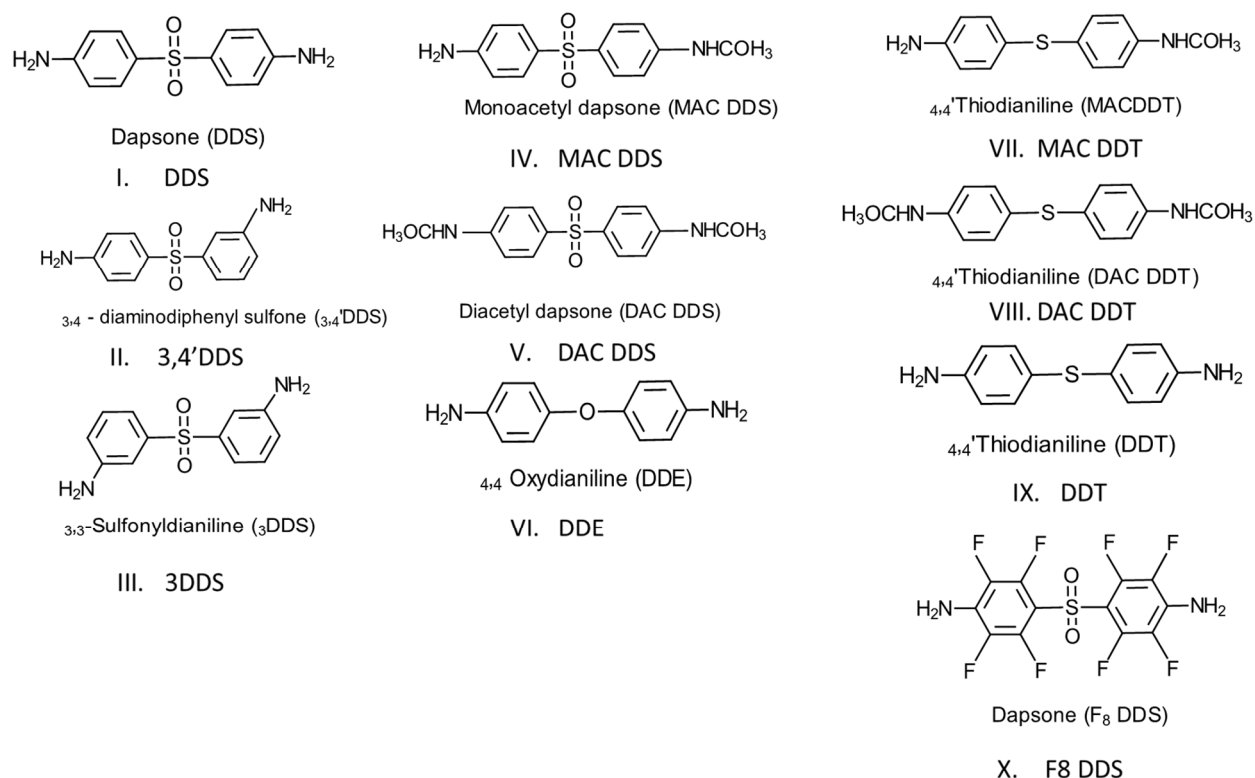


Figure 2.8 Structure of 10 DDS analogues : I. DDS, II. 3, 4' DDS, III. 3 DDS, IV. MAC DDS, V. DAC DDS, VI. DDE, VII. MAC DDT, VIII. DAC DDT, IX. DDT, X F8 DDS.

2.1.12 Dapsone and the activation of T-cells

Like most sulfonamides, DDS can cause hypersensitivity reaction that is likely mediated by T-cells (type IV hypersensitivity reaction). T-cells are implicated because of the time delay from beginning therapy to the time of reaction, approximately 4 weeks. The mechanism of T-cell response to DDS is unknown and three pathomechanisms are assumed. According to the hapten theory a small inert molecule could illicit an immune response by first covalently binding directly to an intra- or extra- cellular protein. Whereas the native protein would originally be recognized as “self” and be ignored by the immune system the protein adduct could stimulate a T-cell response. Alternatively, the pro-hapten concept asserts that non-reactive molecules can be metabolized intracellularly and the metabolites then bind to host

proteins, modifying them. Again, when presented to the T-cell receptor peptides driving from these adducts are recognized as abnormal and the T cell becomes activated to illicit an immune response. This response is seen in reactions with sulfamethoxazole and other drugs (Adam et al, 2011). For DDS to activate T-cells via this mechanism, it would need to be metabolized to the nitroso intermediate, which would then react with protein. Finally, in the ‘pharmacological interactions of drugs with immune receptors’ concept, or pi theory, an inert molecule would interact directly with the antigen presenting cell at the major histocompatibility complex. This binding interaction is sufficient to interact with the T- cell receptor to induce a response. The pi model is an interesting candidate for dapsone hypersensitivity as there is a 21 fold increased risk associated with HLA-B*13:01, an allele common in South East Asia and Australian aborigines (Markova et al, 2014).

2.2 AIMS OF THE INVESTIGATION

The pathomechanism of T-lymphocyte activation by DDS and DDS-NO has not been previously investigated. While the hepatic metabolism of dapsone has been well characterized, it is not clear whether the reactive metabolite (DDS-NO) generated within the liver actually circulates to the skin to activate T-cells that cause cutaneous reactions. Alternatively, localized cutaneous generation of protein reactive metabolites might be responsible for these reactions in the skin. The aims of this chapter were:

- Synthesis / characterization of DDS-NO from DDS hydroxylamine.
 - Determination of whether DDS and/or DDS-NO prime naïve T-cells from healthy donors’ and assessment of cross-reactivity.
 - Detection of whether DDS and/or DDS-NO stimulate dendritic cells directly.
- Sulfamthexazole and nitroso sulfamethaxzole were used as controls during the

experiments. The T-cell priming assay conditions were optimised with these compounds (Falkner et al., 2012; Gibson et al., 2017).

2.3 MATERIALS AND METHODS

2.3.1 Study Area and Approval

The study was approved by the Liverpool research ethics committee. Venous blood (100 mL) samples obtained from 3 healthy donors were collected via venipuncture into heparinized vacutainer tubes. Informed written consent was obtained from all donors before the procedure.

2.3.2 Chemicals and reagents

Dapsone and dapsone analogues were purchased from Sigma-Aldrich (UK). Dapsone hydroxylamine was purchased from Dalton Chemical Laboratories Inc. (Toronto, Canada). Tritiated thymidine-(methyl-³H) was obtained from Moravek (California, USA). Analytical grade acetonitrile, HPLC grade distilled water, and HPLC grade methanol were acquired from Fisher Scientific (Loughborough, United Kingdom). Ammonium formate, DMSO, methimazole, phytohemagglutinin (PHA) was obtained from Sigma-Aldrich (Gillingham, Dorset, United Kingdom). Chemiluminescent substrate was obtained from Thermo Scientific (Northumberland, United Kingdom). Luminex was bought from GE Healthcare Life Sciences (Buckinghamshire, United Kingdom). The microplate reader was manufactured by Dynatech Laboratories Inc., (Chantilly, VA, USA).

2.3.3 Culture medium

All type of cells used in this study were grown in RPMI-1640 cell culture medium with required supplementation. T-lymphocytes were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 25 mM HEPES buffer, 2 mM L-glutamine, GM-CSF (800 U/mL), IL-4 (800 U/mL), penicillin (1000 U/ml), and streptomycin (0.1 mg/ml). Dendritic cells were grown in RPMI-1640 medium supplemented with 10% human AB serum, 25 mM HEPES buffer, 2 mM L-glutamine,

penicillin (100 µg/ml), streptomycin (100 U/ml), and transferrin (25 µg/ml). Naïve T-cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine.

2.3.4 Isolation of peripheral blood mononuclear cells (PBMC)

Venous blood was carefully layered on top of lymphoprep (25 ml) and spun in centrifuge at 2000 r.p.m for 25 minutes at 25°C temperature. The buffy coat layer containing PBMC was removed by using a Pasteur pipette and transferred into a new conical tube. Buffy coat was washed in Hanks balanced salt solution (HBSS) twice at room temperature by centrifugation. Cells were counted using a Neubauer haemocytometer (Sigma-Aldrich) with trypan blue (0.2% w/v) exclusion of dead cells under a Leica DME microscope (Leica Microsystems, Milton Keynes). The percentage viability was estimated as follows: percentage viability = (viable cells ÷ total cells) × 100. Percentage viability was measured at ≥95% for all isolations. Cells were used immediately or after 24h, centrifuged and resuspended in freeze mix containing with 10% DMSO and 90% human AB serum. 10⁷ cells/ml per cryovial were then placed in a Mr Frosty freezing container at -80 °C, cells were stored at -150°C for long-term.

2.3.5 Isolation of PMBCs and separation of subsets of T-cells

CD14⁺ monocytes and T-cell populations were separated by using magnetic beads and columns following to the manufacturer's instructions (Miltenyi Biotech; Bisley, UK). CD14⁺ cells were positively selected from total PBMC. For isolation of naïve and memory T-cells, pan negative T-cell separation was performed using an anti-T-cell antibody beads cocktail. CD3⁺ cells were then subjected to positive selection for T_{reg} (CD25⁺) and memory cells (CD45RO⁺). Finally, the fraction that contained naïve T cells were frozen and stored at – 150°C for further use.

2.3.6 Generation of dendritic cells (DCs)

CD14⁺ monocytes isolated from PBMC using magnetic beads and columns were cultured in dendritic cell culture medium supplemented with IL-4 and GM-CSF for 7-8 days. DCs were then matured using LPS and TFN.

2.3.7 Lymphocyte Transformation Test (LTT)

LTT assay was performed on PBMC isolated from healthy volunteers, to exclude any unlikely pre-sensitization prior to the T-cell priming strategies, using an established protocol (Nyfeler and Pichler 1997). Briefly, PBMC (1.5×10^5 cells, 100 μ L) were cultured in a 96-well U-bottom cell culture plate in triplicate with either 100 μ L DDS (0.125-2 mM) or DDS-NO (10-80 μ M) and incubated at 37°C, 5% CO₂ for 5 days. In this assay tetanus toxoid (TT) 5 μ g/ml was used as a positive control while culture medium was the negative control. Sixteen hours prior to harvesting [³H]-thymidine (0.5 μ Ci/well) was added and lymphocyte proliferation was assessed as counts per minute (c.p.m) using a liquid scintillation counter (Wallac microbeta trilux, PerkinElmer, Cambridge, UK). Proliferative responses, counts per minute (cpm) were converted to a stimulation index (SI) representing the cpm in drug treated cultures divided by the cpm in medium control.

2.3.8 Priming of naive T cells to DDS and DDS-NO.

To perform the T-cell priming assay, dendritic cells were plated (0.8×10^5 cells per well) and co-cultured at 37 C° with naive or memory CD3⁺ T-cells (2.5×10^6 cells per well; 48 well plate) in the presence of either DDS (1 mM) or DDS-NO (40 μ M) for 8 days. The culture period was sometimes extended to four weeks. T-cell cultures were supplemented with IL-2 (5 μ g/mL) every 5 days. T-cell culture was assessed using two methods, [³H]-thymidine proliferation and interferon gamma (IFN- γ) secretion in the presence of either DDS (0.125-2 mM) or DDS-NO (10 - 40 μ M).

2.3.9 [³H]-Thymidine incorporation proliferation and IFN- γ secretion assays.

For the analysis the cell proliferation by [³H]-thymidine incorporation, primed T-cells (1×10^5 ; 200 μ l) were harvested and stimulated with autologous dendritic cells (4×10^3) in the presence of either DDS (1 mM) or DDS-NO (40 μ M). After 48 hours, [³H]-thymidine (0.5 μ Ci/well) was added to the culture plate. Incorporated thymidine was counted after a further 16 hour incubation using a MicroBeta TriLux 1450 LSC β -counter (Perkin Elmer, Cambridge, UK). ELISpot was used, according to the manufacturer's instructions (Mabtech, Nacka Strand, Sweden) to visualize IFN- γ secretion after co-culture of the primed T-cell with dendritic cells and either DDS or DD-NO under the condition described above.

2.3.10 ELISpot Assay

ELISpot plate wells were coated with 100 μ L of high affinity monoclonal antibody IFN- γ (15 μ g/ml) and incubated overnight at 4°C. Plates were washed with HBBS and blocked with T-lymphocyte culture medium for 30 minutes at room temperature. The culture medium was aspirated and naïve T-cells (1×10^5 , 100 μ L) and autologous DCs (1.6×10^5 , 50 μ L) were plated out to a total volume of 200 μ L/well. Cells were cultured in the presence of either DDS (0.125-0.5mM) or DDS-NO (10-40 μ M, 50 μ L) and incubated at 37°C, 5% CO₂ for 48 hours. Cells were discarded after 48 hours and the wells were washed five times with 200 μ L PBS. For the detection of secreted cytokine, biotin-labelled detection antibody was diluted to 1 μ g/ml in PBS containing 0.5% FBS and 100 μ l was added to each well. The plate was incubated at room temperature for 2 hours and the wells were again washed five times with PBS. Streptavidin-ALP diluted PBS containing 0.5% FBS (1:1000) was added to each well and incubated for 1 hour at room temperature. The wells were then washed five times with PBS (200 μ L), and BCIP/NBT substrate (100 μ L/well) was added and incubated in the dark for 15 minutes at room temperature. Finally, wells were inspected for the development of spots and then washed under slow running tap water and left to air dry. ELISpot signal was counted using an AID ELISpot reader (Cadama Medical, Stourbridge, UK).

2.3.11 Synthesis of Nitroso-Dapsone.

DDS-NO was synthesized from DDS hydroxylamine using iron (III) chloride (Figure 2) according to the method of Naisbitt et al. Briefly, DDS hydroxylamine (60 mg, 0.227 mmol) was dissolved in absolute ethanol (10 mL) and added to a stirred solution of iron (III) chloride hexahydrate (0.5g, 1.85 mmol) in distilled water (10 mL) over a period of 5-10 min. The reaction mixture was stirred at room temperature for 5 min and a yellow precipitate was formed. The mixture was filtered under vacuum and the yellow solid product was analysed for purity using LC-MS/MS. A solution for analysis was prepared immediately by ten-fold dilution with acetonitrile. The LC-MS/MS equipment was a 1260 Infinity LC system (Agilent Technologies, Waldbronn, Germany) connected to a 4000 Qtrap (Sciex, Warrington, United Kingdom). An aliquot (3 μ L) was eluted from an Agilent Eclipse XDB-C18 column (3.5- μ m; 150 \times 4.6 mm) at 0.5 mL/min with ACN-formic acid (0.1%, v/v) in formic acid (0.1%, v/v): 25% to 75% over 10 min; 75% for 5 min; 75% to 25% over 0.1 min. Full scanning positive-ion mass spectra were acquired over 5 s between m/z 100-1,000. The ionspray voltage was 5.5 kV; desolvation potential, 120 V; source temperature, 500 $^{\circ}$ C.

2.3.12 Mass spectrometric analysis of DDS-NO binding to glutathione.

DDS-NO (10 mM) was incubated with glutathione (10 mM) in potassium phosphate buffer (10 mM, pH 7.4) at 37 $^{\circ}$ C for 16 h. The resulting crude products were diluted with 0.1% formic acid (1:10 dilution) and analysed by LC-MS/MS as described previously (Meng et al. 2016). Samples were delivered into a 4000 QTRAP mass spectrometer (AB Sciex, Framingham, MA,) coupled with a 1260 Infinity Quaternary Pump HPLC system (Agilent Technologies, Santa Clara, CA) and Kinetex C18 column (2.6 μ m C18, 100 mm \times 2.1 mm, Phenomenex, Macclesfield, Cheshire, U.K.). A gradient from 1% acetonitrile /0.1% formic acid (v/v) to 50% acetonitrile /0.1% formic acid (v/v) in 12 min was applied at a flow rate of 150 μ L/min. Full scanning positive-ion mass spectra were acquired over 5 s between m/z 60 and 1000. The

ionspray voltage was 5.5 kV, desolvation potential was 120 V, and source temperature was 500 °C. Data were analyzed using Analyst software, version 1.5.1 (AB Sciex).

2.3.13 Luminex assay

Luminex is similar to the ELISPOT assay but has a capacity to detect multiple cytokines in the same sample (Beeler and Pichler 2006; Chen et al. 2009; Elsheikh et al. 2011). This assay utilises beads with known spectral characteristics that are bound to particular capture antibodies in order to detect and quantify cytokines secreted by defined cell populations.

Table 1: Summary of the steps involved in the Luminex assay.

Beads	<ul style="list-style-type: none"> • Prewet plate with 100 µL bio-plex buffer per well • Add 50 µL coupled beads per well
stander and sample	<ul style="list-style-type: none"> • Add 50 µL stander / sample / blunk • Incubate (dark , shaking) 30 min
Detection Antibody	<ul style="list-style-type: none"> • Wash 3X with 100 µL bio-plex buffer per well • Add detection antibody 25µ / well • Incubate (dark , shaking) 30 min
streptavidin-PE	<ul style="list-style-type: none"> • Wash 3X with 100 µL bio-plex buffer per well • Add 50µL streptavidine-PE • Incubate (shake) 10 min
Resuspend beads	<ul style="list-style-type: none"> • Wash 3X with 100 µL bio-plex buffer • Resuspend with 125µL bio-plex per well
Read plate	<ul style="list-style-type: none"> • Read plate on bio-plex

2.3.14 Sample preparation.

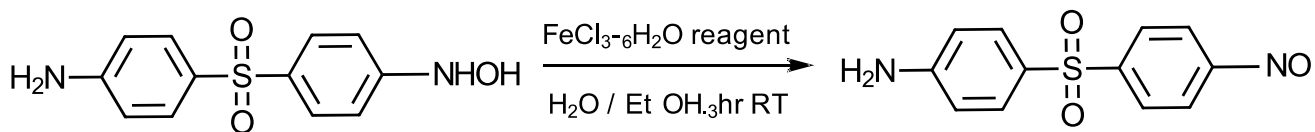
Magnetic beads and columns were used to isolate CD14⁺ monocytes from PBMC (Miltenyi Biotech; Bisley, UK). CD14⁺ cells were cultured in dendritic cell culture medium. This medium was supplemented with GM-CSF (800 U/mL) and IL- 4 (800 U/mL). Cells were co-cultured for 7 days at 37C°, 5% CO₂. Dendritic (0.5x10⁶) were incubated with different concentration of drugs under study; DDS (0.125-1mM), sulfamethoxazole (SMX) (0.125-

1mM), DDS-NO (5-40 μ M), SMX-NO (5-40 μ M). All conditions were conducted in duplicate wells at 37C°, 5% CO₂ for 24 hr. Supernatants were collected (500 μ L) in Eppendorf tubes. After centrifugation at 1400g for 5 minutes to extend cell debris. 200 μ L of supernatant were then stored at -80C°.

2.4 Results

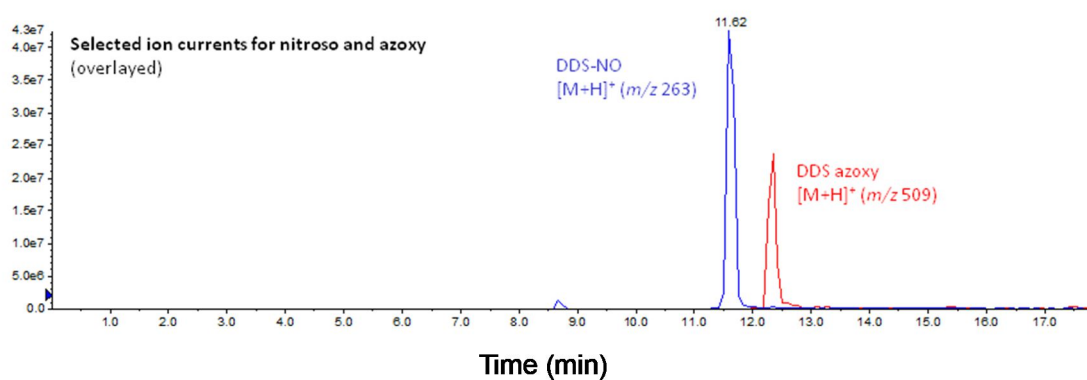
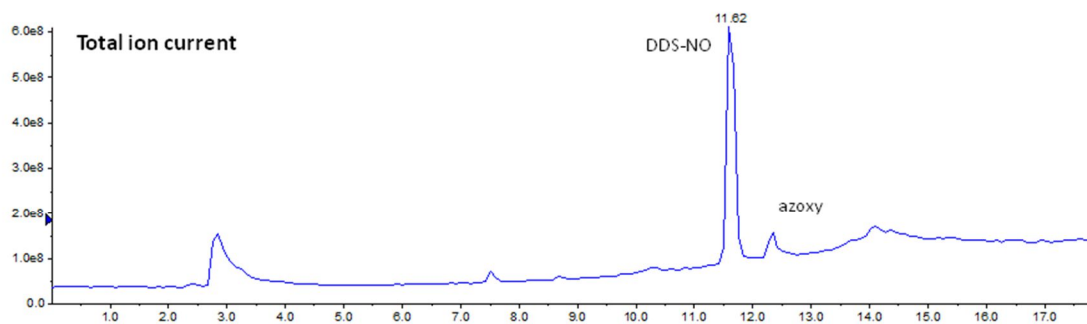
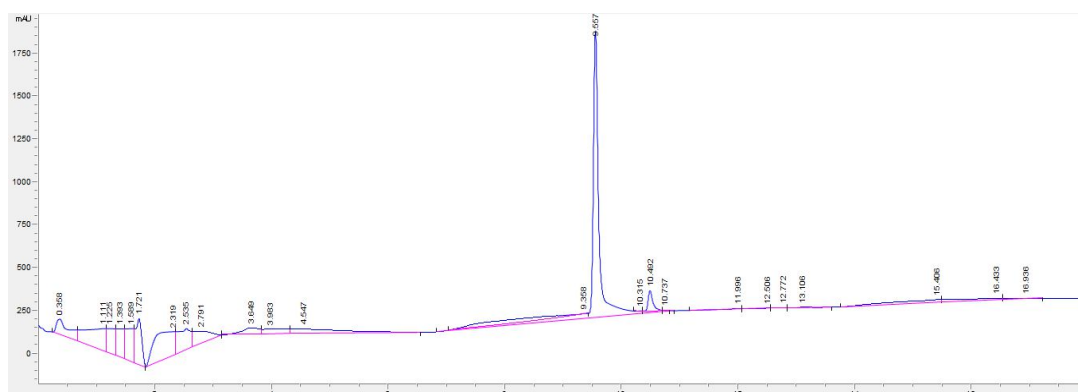
2.4.1 Synthesis and characterization of DDS-NO

A solution of DDS-NO (Figure 2.8A) was prepared and analysed immediately by HPLC and mass spectrometry. The purity of DDS-NO was 95% based on HPLC analysis (Figure 2.9B). DDS-NO (retention time, 11.6 min) was detected as $[M+H]^+$ at m/z 263 (Figure 2.9B). Abundant and characteristic fragment ions were seen at m/z 233 ($[M+H-NO]^+$) and m/z 93 ($[PhO]^+$). The material, typical for an aryl nitroso preparation, contained a small amount of the azoxy derivative ($[M+H]^+$ at m/z 509; retention time, 12.5 min), which does not bind covalently to protein. The reactivity of DDS-NO with the low molecular weight thiol glutathione was confirmed by mass spectrometry (Figure 2.9C). Direct addition of glutathione to DDS-NO resulted in a sulfinamide product, which can be further oxidized to form a sulfonamide and an N-hydroxysulfonamide adduct. Only two of the three putative DDS-NO/glutathione reaction products were detected by LC-MS/MS, namely the [2O] (m/z 586.3) and [3O] N-S conjugates (m/z 602.3) (Figure 2.9C&D). The product ion spectra provided confirmative evidence for the formation of the proposed sulfonamide and N-hydroxysulfonamide adducts shown in Figure 2.10.

Chemical synthesis nitroso-dapsone from dapsone hydroxylamine.**A.****B.**

Dapsone Hydroxylamine

Nitroso-Dapsone



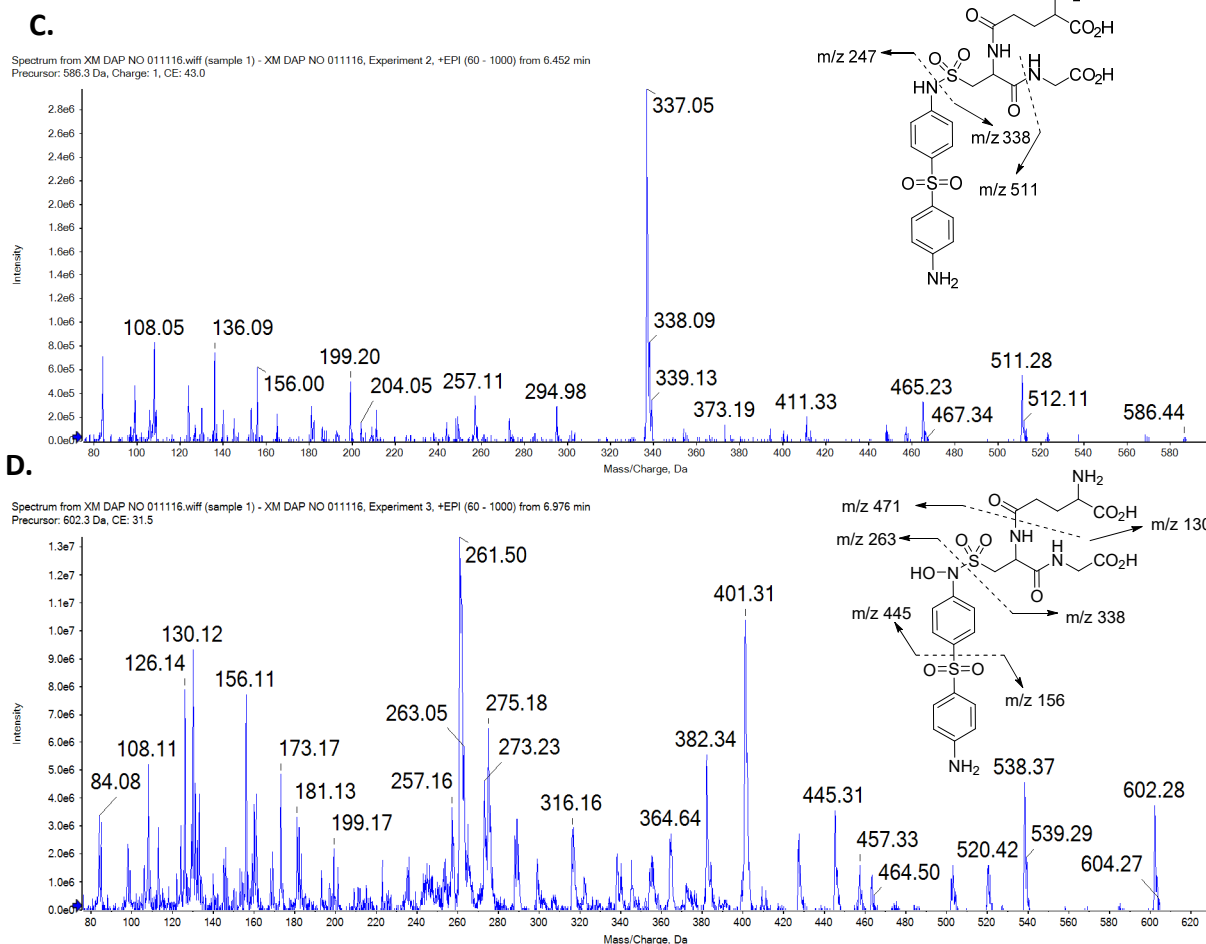


Figure 2.9: Synthesis of DDS-NO from DDS hydroxylamine. DDS hydroxylamine is converted to DDS-NO in the presence ethanol and iron chloride hexahydrate. The yellow solid paste was filtered analysed by LC-MS/MS for purity. The DDS-NO fraction as formed at 11.8 min retention time. Anticipated pathways of modification DDS-NO to cysteine residues of glutathione is shown. Mass increments resulting from adduction of reactive metabolite (DDS-NO) to GSH are involved.

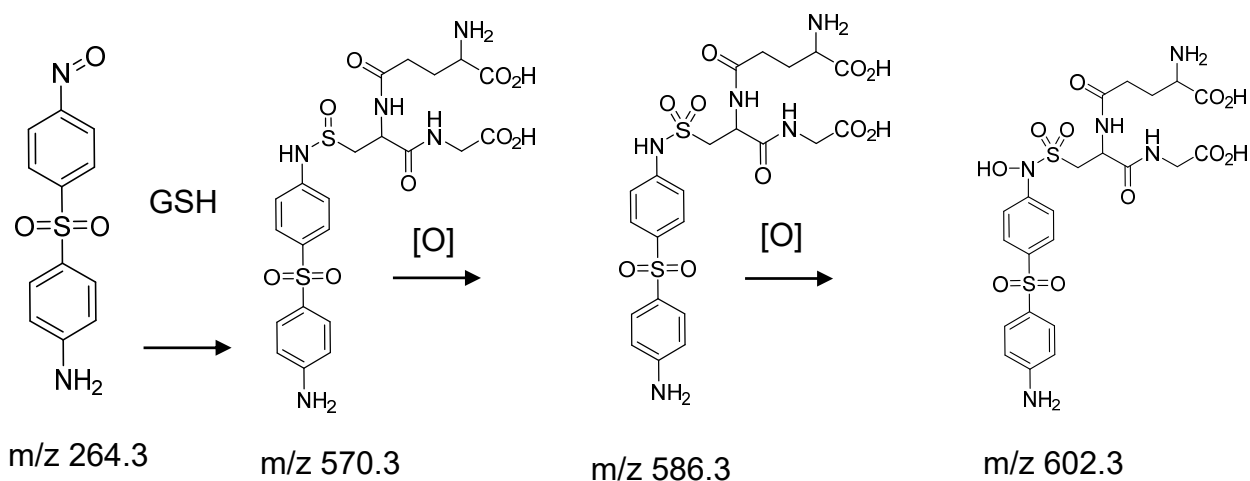


Figure 2.10: Scheme showing chemical structure of DDS-NO hapten adduct formed through covalent binding of DDS-NO to glutathione. DDS-NO haptenate the protein and peptides by nucleophilic attack at cysteine residues, resulting in a mass increases of 570,586 and 602 amu for each modified cysteine residue. The chemistry is complicated by the methods conventionally used to process proteins for mass spectrometry analysis, namely iodoacetylation of cysteine. The drug itself was shown to be iodoacetylated.

2.4.2 Toxicity assay

To determine the toxic profiles of tested compounds, PBMC were incubated with graded concentrations of DDS, DMSO, DDS-NHOH, DDS-NO and SMX-NO in the presence of the mitogen PHA. Thymidine incorporation was used to determine inhibition of cell proliferation (Figure 2.11). DDS inhibited PBMC proliferation at concentration of 1000 μ M. In contrast, DDS-NHOH and DDS-NO inhibited PBMC proliferation at 25 μ M.

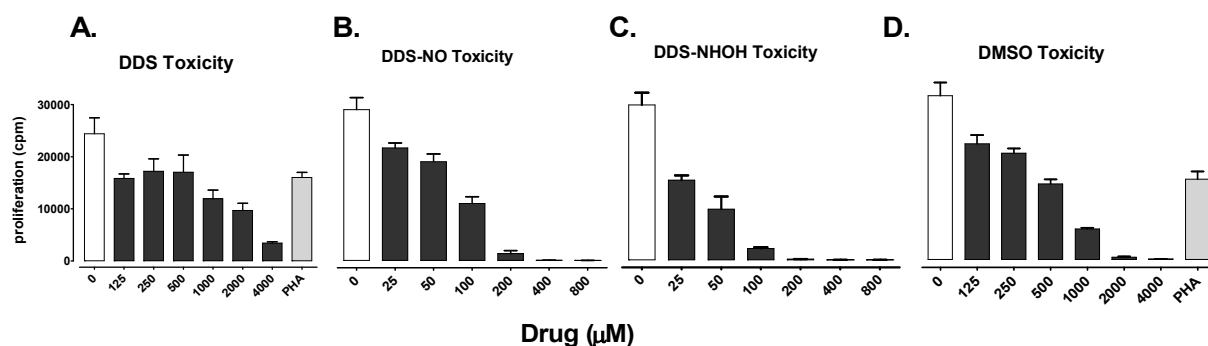


Figure 2.11 Toxicity of DDS and DDS metabolites. PBMC (1.5×10^4 , 100 μ L) were incubated with graded concentration of A) DDS (125 – 4000 μ M), B) DDS-NO (25 – 800 μ M), C) DDS-NHOH (25-800 μ M). PHA as added to stimulate proliferation. 96-Well U-bottom plates were incubated at 37 $^{\circ}$ C and 5% CO_2 for five days. [3]Thymidine was added at final of 16 hr incubation and T-cell proliferation evaluated using scintillation counting.

2.4.3 Lymphocyte transformation test (LTT)

PBMC samples from 3 DDS naïve donors, which were all HLA-B 13:01 negative and evaluated the proliferation of PBMC in the presence of the test compounds. Proliferation of PBMC with DDS and DDS-NO was not observed. In contrast, TT (tetanus toxin), used as a positive control, stimulated PBMC proliferation. (Figure 2.12).

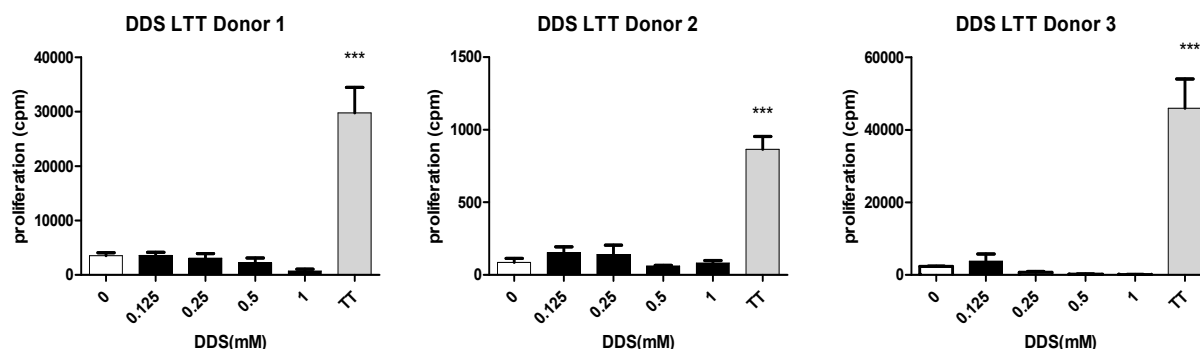
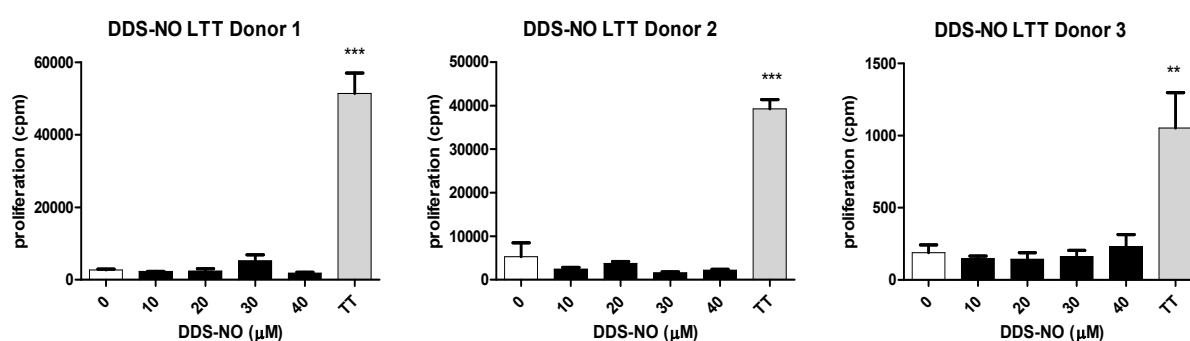
A.**B.**

Figure 2.12 Lymphocyte transformation test (LTT) assay using PBMC from healthy donors not previously exposed to DDS. PBMC (1.5×10^4 cells, 100 μ L) were incubated with graded concentrations of either (A) DDS (0.125–2 mM) or (B) DDS-NO (10–40 μ M) in 96-Well U-bottom plates. Plates were incubated at 37°C, 5% CO₂ for 5 days. [3H]-thymidine (0.5 μ Ci) was added for the final 16 hours of incubation and T-cell proliferation evaluated using scintillation counting.

2.4.4 Priming of naïve T-cells from healthy donors to DDS and DDS-NO.

Naïve T-cell proliferation responses, following priming with DDS or DDS-NO were assessed with T-cells from three healthy donors (Figure 2.12). In order to prime either DDS or DDS-NO-specific T-cell responses, naïve CD45RA⁺ T-cells from HLA-B*13:01-negative volunteers ($n = 3$) were cocultured with autologous DCs in the presence of DDS or DDS-NO. After 8 days the primed T-cells were restimulated with fresh DCs and the drug and antigen-specificity was assessed using [3] thymidine and ELISpot. Moderate levels of proliferation and IFN- γ release with both DDS and DDS-NO was detectable and the response was found to be antigen-specific

and dose-dependent (Figure 2.13). T-cells from donor 3 crossreacted with the alternative form of the DDS antigen.

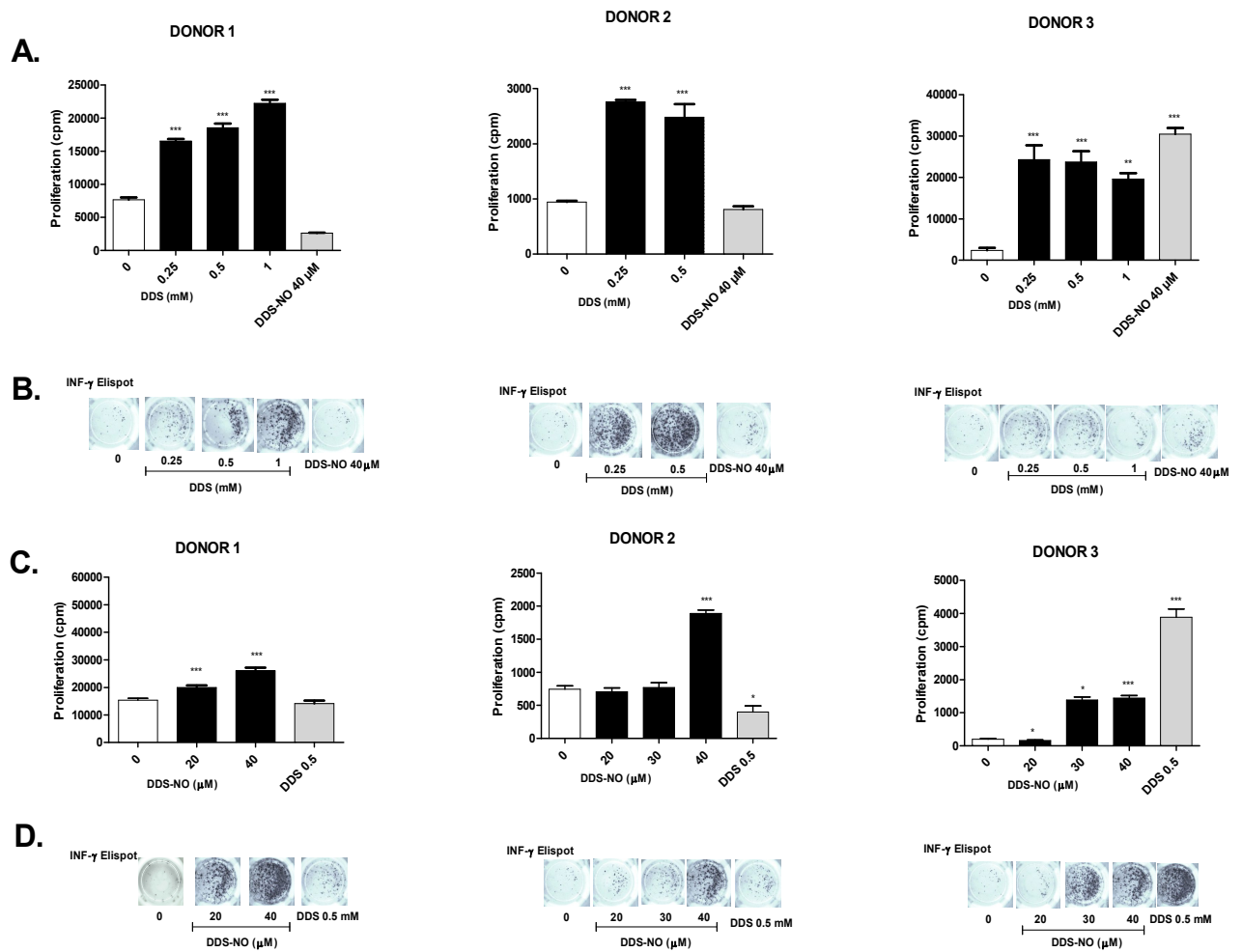


Figure 2.13- DC-priming of naïve T-cells to DDS and DDS-NO. A, C) Naïve T-cells from 3 donors were co-cultured with monocyte-derived DC and either DDS (0.5mM) or DDS-NO (40 μ M) for 7 days. Afterwards, fresh autologous DC were cultured with the primed T cells in the presence of graded concentration of DDS-NO (10–40 μ M) or DDS (0.125–1 mM). Plates were incubated at for 48 hours at 37°C, 5% CO₂. [³H]Thymidine was added during the final 16 h of incubation and T cell proliferation evaluated by using scintillation counting. B, D) Cells were cultured as described above and INF- γ ELISPOT performed according to manufacturer's instructions.

2.4.5 Cytokine production by DC cultured with DDS, SMX, DDS-NO or SMX-NO.

Drugs were incubated for 24-hours with DCs and, supernatant (200 μ L) was assessed using luminex according to the manufacturer's protocol. Cytokines were detected, using serial dilutions of the standards of all cytokines for quantification. IL-8, IL-13, IL-15, IL-17 and TNF- α and MIP-1b are pro-inflammatory cytokines, while IP-10, and G-CSF are anti-inflammatory. (Table 2.2)

Table 2.2: Cytokines production of DC cocultured with Drug.

	Cytokines							
Drug	IL-8	IL-13	IL-15	IL-17	TNF-α	MIP-1b	IP-10	G-CSF
DDS	(+)	(-)	(-)	(-)	(-)	(-)	(+)	(-)
DDS-NO	(+)	(-)	(-)	(-)	(+)	(+)	(+)	(-)
SMX	(+)	(-)	(-)	(+)	(+)	(-)	(+)	(-)
SMX-NO	(+)	(+)	(+)	(-)	(+)	(+)	(-)	(+)

2.5 DISCUSSION

Understanding of the patho-mechanism of immunological drug reactions remains an important challenge (Uetrecht and Naisbitt, 2013). Due to their complexity they are burden on health care systems (Davies et al., 2009) and an impediment to the drug development process. Studies show that there are a number of factors involved in hypersensitivity which include genetic and environmental factors and the chemical properties of drugs (Knowles et al. 2002; Macy 2004). DDS undergoes N-acetylation in the liver which leads to the generation of non-toxic metabolites which can then be safely eliminated from the body via urine (Zuidema et al., 1986; Gelber et al., 1971). DDS also undergoes CYP-450-mediated metabolism to yield DDS hydroxylamine (DDS-NHOH) (Ganesan et al., 2010). DDS-NHOH is not protein-reactive and can be excreted through the urine in an unchanged state (Bluhm et al., 1999). Rieder and his colleagues in 1988 used a cell based assay to show that DDS-NHOH is toxic, inducing cell death at relatively low concentrations. This toxicity is thought to be due to DDS-NHOH (Rieder et al., 1988). DDS-NHOH undergoes auto-oxidation and forms a highly protein-reactive DDS-NO intermediate (Coleman, 1995). DDS-NO-modified proteins can be degraded into peptide fragments and presented in an MHC class II-restricted manner to CD4⁺ T-lymphocytes or MHC class I-restricted manner to CD8⁺. If this is the case, DDS-NO would be the primary trigger of hypersensitivity reactions.

DDS is structurally similar to sulphonamides such as SMX (Brackett et al., 2004). SMX also forms a nitroso metabolite and there are number of studies which show that SMX-NO act as trigger of immune responses in human and animals. Despite this, complimentary studies have shown SMX, the parent drug, also interacts directly with MHC molecules to activate T-cells (Naisbitt et al. 2001; Uetrecht and Naisbitt 2013). There is a gap in literature regarding the pathomechanism of DDS hypersensitivity in terms of the relationship between the DDS-NO formation and T-cell activation. Studies conducted on normal human dermal fibroblasts and

normal human epidermal keratinocytes found that DDS hydroxylamine metabolites are formed in skin cells and this leads to adduct formation. This suggests that adduct are formed at the site of the adverse event, (Bhaiya et al, 2006 ; Vyas et al,2006) but studies describing the relationship between DDS metabolism and T-cells activation have not performed. There are also number of studies which have focused on the role of HLA alleles in drug hypersensitivity reactions by different drugs (Illing et al., 2012; Yun et al., 2012). Zhang et al, demonstrated that HLA-B*13:01 allele acts as a risk factor of DDS hypersensitivity syndrome (Zhang et al, 2013). This suggests that binding of the drug antigen (s) to this MHC molecules might be important for T-cell activation. To investigate this we synthesized DDS-NO by oxidation of DDS-NHOH in laboratory. Inhibition of PBMC proliferation was performed to determine the optimum drug concentrations for the experiments such as priming of naïve T-cells and cross reactivity. DDS-NHOH was converted to DDS-NO using ethanol and iron chloride hexahydrate. The yellow solid paste had a purity of 95%. DDS-NO was incubated with glutathione to characterize the adduct structures formed as nitroso compounds are known to bind selectively to thiol groups. Similar to SMX-NO, direct addition of glutathione to DDS-NO resulted in formation of a sulfinamide product, which was detected after 1 minute incubation (Callan et al. 2009). However, the sulfinamide is unstable and can be further oxidized to form a stable 2[O] adduct and an N-hydroxysulfonamide adduct. Indeed, two stable adducts were detected after incubation of DDS-NO with glutathione. The structure of 2[O] adduct has been controversial, however, mass spectrometric data from this study provided evidence for formation of the sulfonamide, which was presumably derived from N-hydroxysulfonamide (see figure 2.9 C&D)

For determination of the optimized priming doses of both DDS and DDS-NO, the inhibition of PBMC proliferation was assessed. The toxicity assay revealed that DDS-NHOH and DDS-NO are highly toxic, when compared with DDS. The optimized non-toxic dose for DDS was

between 0.2-0.5 mM while the optimized dose for DDS-NO was (20 μ M). The LTT assay was then performed to confirm that naïve healthy donor PBMC are not stimulated to proliferate with parent drug or DDS-NO. PBMC from all 3 donors were not activated with DDS or DDS-NO which indicate that there are no DDS or DDS-NO specific T-cells circulating in their peripheral blood.

The *in vitro* priming assay model has the ability to explore the immune stimulatory capability of drug antigens in naïve donors (Faulkner et al. 2012). In our study naïve T-cells were primed to either DDS or DDS-NO. The assay involved an initial co-culture period of naïve T-cells, DCs and DDS or DDS-NO for 7-8 days. The resulting data from [3H] thymidine proliferation and IFN- γ secretion assays revealed that naïve T-cells were sensitized to DDS and DDS-NO. Drug-primed naïve T-cells were stimulated to proliferate in a dose-dependent manner (Figure.2.13). Furthermore, cross reactivity was observed with 1/3 donors (figure 2.13).

Cytokines play very important role in T-cell reactions. These are soluble proteins that are secreted by cells of the immune system. The main function of these cytokines is to alter the behavior and properties of different cell types within and outside the immune system. Cytokines produced by DCs impact on the polarization of antigen-specific T-cells deriving priming thus, we assessed the cytokines secreted from DCs following exposure to DDS and DDS-NO.

There are a number of ways the cytokines can be measured in biological samples. These include secreted bioassays, enzyme-linked immunosorbent assays (ELISA), radioactive immunosorbent assays, microarrays and most recently, multiplex assays. In this study, a luminex assay was performed on dendritic cell supernatant, 24hr after DDS and DDS-NO treatment. The compounds did not influence the secretion of most cytokines (Table 2.2), TNF-

Chapter 2

a , IP-10 , IL-8, MIP-1b, IL-17,IL-13,IL-15 and G-CSF was observed in the presence of DDS and/or DDS-NO, SMX and SMX-NO (Table 2.2).

In conclusion, this first piece of work shows that it was possible to synthesize DDS-NO and assess its immunogenicity. Both DDS and DDS-NO primed naïve T-cells from healthy donors. Subsequent chapters describe the generation of DDS and DDS-NO-specific T-cell clones and characterization of their phenotype and functionality.

Chapter 3: Characterisation of Dapsone and nitroso-dapsone -specific T-cell clones from 3 HLA-B*13:01 negative healthy volunteers.

3.1 Introduction:	88
3.2 Materials and Methods	91
3.2.1 Chemicals and reagents	91
3.2.2 Culture medium	91
3.2.3 Isolation method of peripheral blood mononuclear cells (PBMC)	91
3.2.4 Generation of drug-specific T-cell clones	92
3.2.5 Characterization of dapsone – specific TCCs	93
3.2.6 ELISpot Assay	93
3.2.7 Antigen presenting cell (APC) fixation and antigen pulsing assays	94
3.2.8 MHC restriction assay	94
3.2.9 T-cell phenotyping (Flow cytometry)	95
3.2.10 Graphs and Statistics	95
3.3 Results:	96
3.3.1 Generation of drug-specific T- cell clones	96
3.3.2 T-cell CD phenotyping	100
3.3.3 The effect of glutathione on the activation of DDS and DDS-NO specific TCC	100
3.3.4 Characterisation of the mechanism of activation of DDS and DDS-NO-specific TCC	101
3.3.5 Drug-specific TCC secrete a mixture of Th1, Th2 and cytokines molecules	103
3.3.6 DDS-specific TCC clone cross reactivity to analogues DDS or sulfonamides	104
3.4 Discussion	106

3.1 Introduction:

Drug-specific T-lymphocytes participate in the pathogenesis of drug hypersensitivity reactions through a variety of means including the secretion of cytokines and cytolytic mediators. Despite significant advances in recent years, definition of the pathway(s) of drug antigen presentation that result in T-cell activation remains a subject of debate. Drugs can activate T-cells via a hapten pathway involving the formation of protein adducts (Meng et al. 2017; Padovan et al. 1997; Brander et al. 1995). The hapten response is dependent on protein processing within antigen presenting cells and the generation of an antigen that comprises of the MHC binding peptide and the drug moiety bound covalently to a specific nucleophilic amino acid. Drugs might also covalently modify MHC molecules or peptides embedded within MHC molecules on the surface of antigen presenting cells to activate T-cells. In addition to this, drugs have been shown to interact with MHC molecules and T-cells directly via readily reversible bonds (pharmacological interaction pathway) (Schnyder et al. 2000; von Greyerz et al. 1999; Ko et al. 2011; Watkins & Pichler 2013). Although the precise nature of the pharmacological interaction remains ill-defined several drugs have been shown to stimulate T-cells via this pathway (Wu et al. 2007; Burkhart et al. 2001; Yun et al. 2014). Finally, the nucleoside reverse transcriptase inhibitor abacavir activates T-cells by binding deep within the peptide binding groove of one specific MHC molecule, HLA-B*57:01, altering the 3-dimensional space and hence the nature of the peptides that subsequently bind (Illing et al. 2012; Ostrov et al. 2012; Norcross et al. 2012). It has been proposed, although not yet proven, that these altered peptides and not the drug *per se* activate the T-cells involved in abacavir hypersensitivity.

Knowledge of the drug MHC binding interaction is complicated by the fact that most drugs undergo extensive metabolism and one or more metabolites might activate T-cells via any of

the pathways described above. Furthermore, our ability to study drug metabolite-specific T-cell responses is limited by: (1) the almost complete absence of synthetic drug metabolites for functional studies; and (2) a disconnect between the drug protein binding interactions that occur in patients and *in vitro* in cell culture systems. To date, researchers have shown that T-cells isolated from patients with (1) allopurinol-induced hypersensitivity are preferentially activated with the active metabolite oxypurinol through a direct binding interaction with HLA-B*58:01 (Yun et al. 2013); and (2) sulfamethoxazole-induced hypersensitivity reactions are activated with the cysteine-reactive nitroso metabolite through a hapten mechanism (Schnyder et al. 2000; Castrejon et al. 2010).

Given the limited availability of synthetic reactive metabolites, the aim of this study was to focus on dapsone (DDS) and the synthetic metabolite nitroso-dapsone (DDS-NO) to investigate the molecular mechanism(s) of antigen-specific T-cell activation using cells cloned from healthy volunteers. Cytochrome P-450, flavin monooxygenase and peroxidase-mediated *N*-hydroxylation results in the formation of DDS hydroxylamine (Zuidema et al. 1986; Roychowdhury et al. 2007; Piyush M Vyas et al. 2006; Piyush M. Vyas et al. 2006). DDS hydroxylamine is susceptible to auto-oxidation and the derived nitroso species has been shown to bind covalently to protein (Roychowdhury et al. 2007; Bhaiya et al. 2006; Roychowdhury et al. 2005). Despite this, the nature of drug or drug metabolite interaction with T-cells has not been defined.

Although DDS is usually well tolerated and suitable for long term treatment, a severe hypersensitivity syndrome with a mortality rate of 9.9% develops in 0.5-3.6% of patients (Kosseifi et al. 2006). Reactions are characterised by a delayed onset (usually 4-6 weeks) with a long latent period (Kosseifi et al. 2006; Agrawal & Agarwalla 2005; Prussick & Shear 1996).

Chapter 3

Zhang et al found that HLA-B*13:01 expression is a predictor of the hypersensitivity syndrome in Chinese patients with leprosy (Zhang et al. 2013). These genetic data suggest that the adaptive immune system may be involved in the disease pathogenesis; however, the activation of T-cells with dapsone and dapsone metabolites has not been studied. Therefore the aim of this chapter was to generate DDS- and DDS-NO specific T-cell clones from healthy volunteers and investigate the molecular pathomechanism of dapsone hypersensitivity.

3.2 Materials and Methods

3.2.1 Chemicals and reagents.

DMSO, dapsone, phytohemagglutinin (PHA) and all the dapsone analogues used in this study were purchased from Sigma-Aldrich (UK). Dapsone hydroxylamine was purchased from Dalton Chemical Laboratories Inc. (Toronto, Canada). Tritiated thymidine-(methyl-³H) was obtained from Moravsek (California, USA). Analytical grade (Analar) acetonitrile, HPLC grade distilled water, and HPLC grade methanol were acquired from Fisher Scientific (Loughborough, United Kingdom).

3.2.2 Culture medium.

All type of cells used in this study were grown in RPMI-1640 cell culture medium with required supplementation of human serum albumin. T-lymphocytes were grown in RPMI-1640 medium supplemented with 10% human AB serum, 25 mM HEPES buffer, 2 mM L-glutamine, penicillin (1000 U/ml), streptomycin (0.1 mg/ml), and transferrin (25 µg/ml). Dendritic cells were grown in RPMI-1640 medium supplemented with 10% human AB serum, 25 mM HEPES buffer, 2 mM L-glutamine, penicillin (100 µg/ml), streptomycin (100 U/ml), GM-CSF (800 U/ml), IL-4 (800 U/ml) and transferrin (25 µg/ml).

3.2.3 Isolation method of peripheral blood mononuclear cells (PBMC).

PBMC were isolated from venous blood collected in heparinized vacutainer tubes. Blood was centrifuged at 2000 rpm for 25 minutes at 25°C temperature. The buffy coat layer containing PBMC was removed by using a Pasteur pipette and transferred into a new tube. Buffy coat was washed in Hanks balanced salt solution (HBSS) two times at room temperature by centrifugation. Cells were then counted using a Neubauer haemocytometer (Sigma-Aldrich) under a Leica DME microscope (Leica Microsystems, Milton Keynes).

3.2.4 Generation of drug-specific T-cell clones

Naïve T-cell from healthy volunteers were primed to either DDS (0.5 mM) or DDS-NO (20 μ M). As described in chapter 2, T-cells were then cloned by serial dilution. Briefly, T-cells were plated at 0.3, 1 and 3 cells per well in a 96 well culture plate. The T-cells were then stimulated with cocktail of irradiated allogeneic PBMC (5×10^6) and PHA (20 μ L/ml) in a total volume of 200 μ L. IL-2 was added every 2 days to maintain the proliferative response. After 14 days the procedure was repeated and viable clones were split into 4 wells of fresh 96 well plate prior to testing antigen specificity. Epstein-Barr virus (EBV) transformed B-cell lines were generated by transformation of freshly isolated PBMC using supernatant from the virus-producing cell line B95.8. 5ml of the supernatant was filtered by a 0.45 μ m syringe filter on to a PBMC pellet containing 5×10^6 cells. 5 μ L of cyclosporin A (CSA) was then added to prevent T-cell mediated suppression of B-cell infection and to inhibit EBV from inducing T-cell proliferation. After overnight incubation (37°C, 5% CO₂), cells were washed, resuspended at 1×10^6 /ml in antigen-presenting cell medium containing CSA (1 μ g/ml) and transferred to a 24-well cell culture plate (1ml/well). Cells were fed twice per week with fresh antigen-presenting cell medium and CSA was omitted from the culture medium after 14 days. When confluent populations were observed, the cells were transferred to a tissue culture flask. Autologous Epstein-Barr virus (EBV)-transformed B-cell lines were used as APC in assays involving T-cell clones (TCCs). Antigen-specificity was assessed by culturing irradiated EBV-transformed B cells (1×10^4 /well) and DDS or DDS-NO with drug-specific T-cell clones (5×10^4 /well; 200 μ L) for 48 hours. Proliferation was measured by the addition of [³H]-thymidine followed by scintillation counting. Clones with a stimulation index (proliferation due to drug/proliferation due to medium) greater than 2 were expanded by repetitive stimulation with irradiated allogeneic PBMC (5×10^5 /well), IL-2 (5 μ g/ml) and PHA (10 μ g/ml).

3.2.5 Characterization of dapsone – specific TCCs

For this experiment, DDS and DDS-NO specific TCC (5×10^4 cells, 50 μ L) were co-cultured with irradiated autologous EBV-transformed B-cells (1×10^4 cells, 50 μ L) in the presence of DDS (0.125-2 mM), DDS-NO (40 μ M) or SMX-NO (40 μ M) in duplicate using a 96-well U-bottom plate. After this, the plates were incubated at 5% CO₂ for 48 hours then [³H]-thymidine (0.5 μ Ci) was added for the final 16 hours of the incubation. Finally, T-lymphocyte proliferation was determined using scintillation counting.

3.2.6 ELISpot Assay

ELISpot plate wells were coated with 100 μ L of a high affinity monoclonal antibody IFN- γ (15 μ g/ml) and incubated overnight at 4°C, according to manufacture instructions. Wells were washed five times with sterile PBS and blocked with 200 μ L of T-lymphocyte culture medium for 30 minutes at room temperature. The culture medium was aspirated out of the well and replaced with naïve T cells (1×10^5), autologous DCs (1.6×10^5) and drug to a total final volume of (200 μ L/well). Cells were then cultured in the presence of either DDS (0.125-0.5mM), DDS-NO (10-40 μ M, 50 μ L) and incubated at 37°C, 5% CO₂ for 48 hours. Cells were discarded after 48 hours and the wells were washed five times with 200 μ L PBS. For the detection of the captured cytokine, biotin-labelled detection antibody was diluted to 1 μ g/ml in PBS containing 0.5% FBS and 100 μ l added to each well. The plate was incubated at room temperature for 2 hours and the wells were again washed five times with PBS. Streptavidin-ALP diluted PBS containing 0.5% FBS (1:1000) was added to each well and incubated 1 hour at room temperature. The wells were then washed five times with PBS (200 μ L), and BCIP/NBT substrate (100 μ L/well) was added and incubated in the dark for 15 minutes at room temperature. Finally, wells were inspected for the development of spots and then washed under slow running tap water and left to air dry. Elispot plates were then viewed using an ELISpot reader (Cadama Medical, Stourbridge, UK).

3.2.7 Antigen presenting cell (APC) fixation and antigen pulsing assays

Antigen presenting cell fixation was used to investigate the role of intracellular metabolism in the activation of T- cell clones. For this, autologous EBV-transformed B-cells (2×10^6 cells/ml) were washed twice in HBSS to exclude FBS and resuspended in HBSS (1 ml). Next, glutaraldehyde (25%, 1 μ L) was added and then the cells were lightly mixed for 30 seconds. After this step, glycine (1ml of 1 M) was rapidly added to the mixture and the cells were mixed for a further 45 seconds. To remove glutaraldehyde, cells were washed five times and then resuspended in T cell culture medium. T cell clones (5×10^4 , 50 μ L) were co-cultured with glutaraldehyde-fixed EBV-transformed B-cells (1×10^4 cells, 50 μ L) in the presence or absence of DDS (0.5 mM) or DDS-NO (20 μ M). In other experiments, antigen presenting cell were pulsed with DDS or DDS-NO for 16 hours and washed extensively with HBSS to exclude free DDS. The pulsed antigen presenting cells were co-incubated with drug-specific T- cell clones in a 96-well plate for 48 hours under 5% CO₂ at 37°C, [³H]-thymidine was added during the last 16 hours incubation and antigen a specific response evaluated.

3.2.8 MHC restriction assay

In order to define whether DDS or DDS-NO presentation to TCCs was MHC class I/II restricted, anti-human MHCI (HLA-A, -B, -C), and anti-human MHC II (HLA-DP, -DQ, -DR) antibodies (5 μ g/mL) were used. In the initial of experiment, autologous EBV-transformed B-cell lines (1×10^4 , 50 μ L) were pre-incubated with either MHC I, MHC II or isotype control blocking antibodies (5 μ g/ml) at 5% CO₂, 37°C for 30 minutes. The antigen presenting cell were then co-cultured with DDS or DDS-NO specific TCC (5×10^4 , 50 μ L) with or without drug for 48 hours. [³H]-thymidine (0.5 μ Ci) was added for the final 16 hours of incubation and T cell proliferation evaluated using scintillation counting. IFN- γ ELISpot was also performed using the same conditions as described a above.

3.2.9 T-cell phenotyping (Flow cytometry)

To determine the CD4 and CD8 phenotype of TCC, flow cytometry was used. T-cell suspensions (100 μ L) were stained with CD4-PE (3 μ L) and CD8-APC (3 μ L) antibodies and incubated at 4°C for 20 minutes in the dark. Cells were washed and resuspended in 200 μ L of FACS buffer and CD4, CD8 expression determined using the FACS Canto™ II system.

3.2.10 Graphs and Statistics

Graphs were produced using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Data analysis and statistical tests were conducted using GraphPad Prism 5. In order to determine statistical significance when comparing two treatment groups, t test was used. In experiments for which more than two treatment groups were compared a one-way ANOVA. Differences that gave p values were considered statistically significant as follows (ns $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.001$, *** ≤ 0.001).

3.3 Results:

Naïve T-cell from healthy donors were primed to DDS and DDS-NO (see chapter 2 section 2.4.4).

3.3.1 Generation of drug-specific T cell clones.

DDS- and DDS-NO-specific TCC were generated from previously primed T-cells of 2 out of 3 donors to investigate pathways of T-cell activation. Initial testing revealed that 9 out of the 92 expanded TCC from DDS-primed T-cells were stimulated to proliferate with DDS (i.e., stimulation index of 2 or more; Figure 3.1 A). Three hundred and two TCC were expanded from the DDS-NO-primed cells. Of these, 24 were stimulated to proliferate with DDS-NO (Figure 3.1B). The drug and drug metabolite-responsive TCC were expanded for subsequent testing. TCC were found to proliferate in a concentration-dependent manner in the presence of DDS or DDS-NO (Figure 3.3). Clones were not activated with DDS or DDS-NO in the absence of antigen presenting cells (Figure 3.2). Furthermore, DDS and DDS-NO specific clones displayed no cross-reactivity with DDS-NO and DDS respectively (Figure 3.4).

Generation of dapsons and nitroso- dapsons-specific TCC.

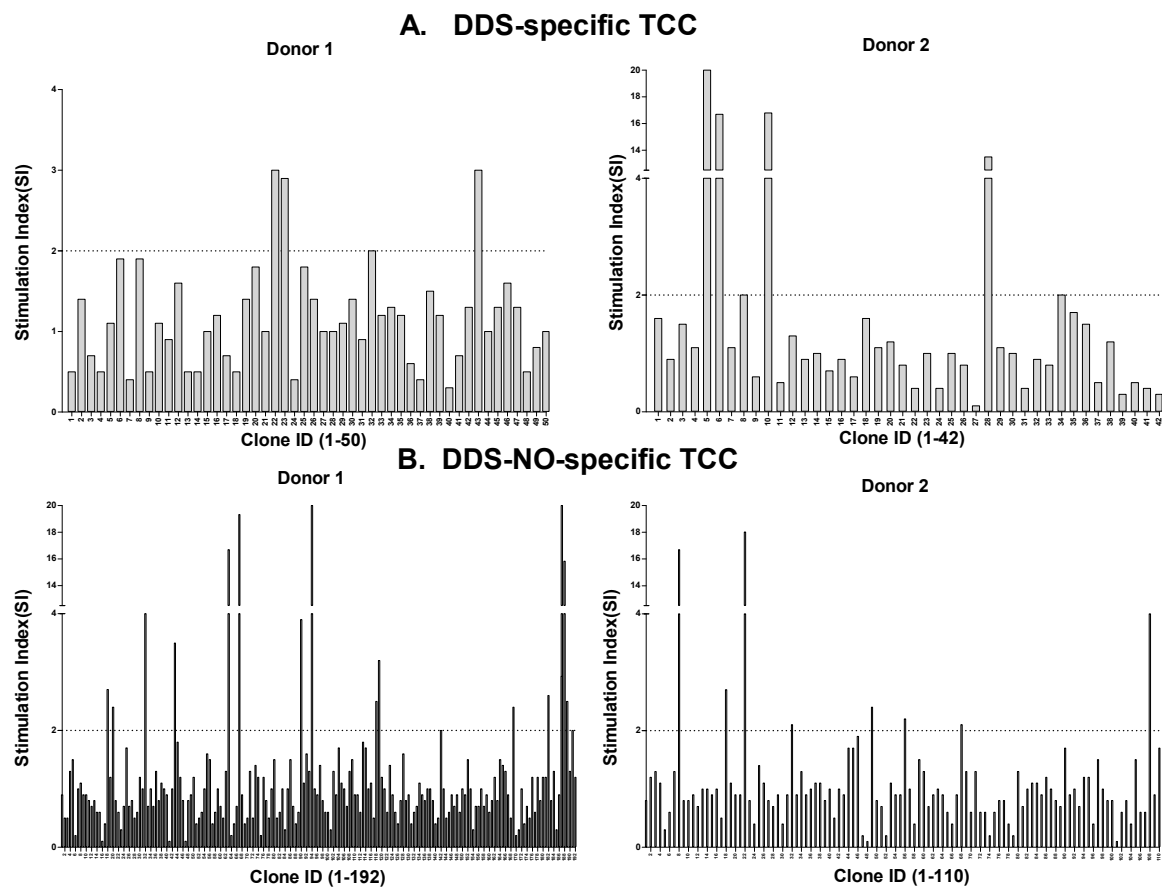
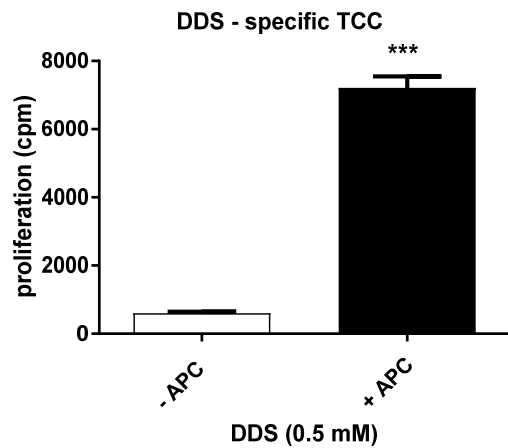


Figure 3.1: Generation of DDS- and DDS-NO-specific TCCs from two drug-naïve volunteers: - TCC were generated using serial dilution method. Clones were co-incubated with irradiated autologous EBV-transformed B cells and DDS or DDS-NO. Culture medium was utilised as negative control. The plates were incubated at 37 °C, 5%CO₂ for 48 h. Radioactive thymidine was added later on during the last 16 h of incubation. Clones with stimulation index > 2 were selected and expanded for further functional assays. A, B) DDS and DDS-NO TCC generated from 2 donors.

Antigen presenting cells are essential for the activation of DDS and DDS-NO –specific TCC.

A



B

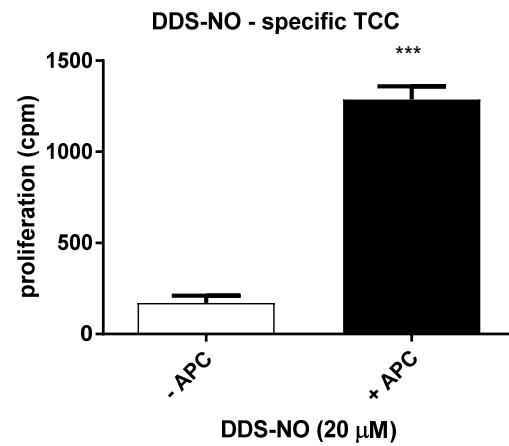
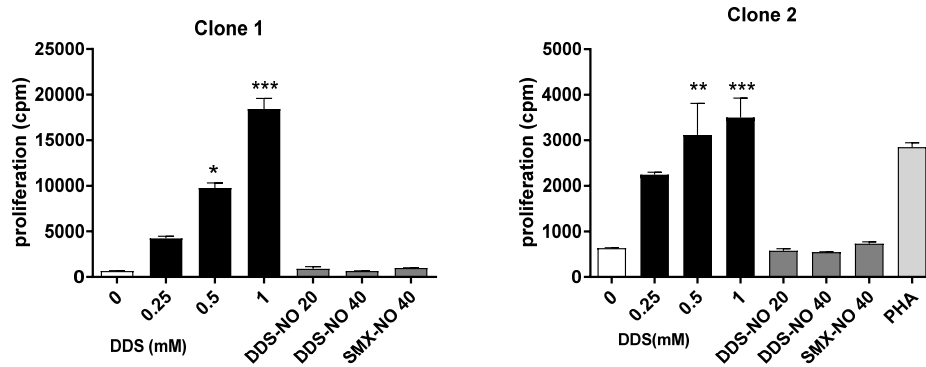


Figure 3.2: DDS and DDS-NO-specific TCC was co-cultured with irradiated autologous EBV-transformed B-cells and DDS in a 96 wells plate and incubated at 5% CO₂ for 48 h. Proliferation was determined using radioactive thymidine incorporation during the final 16 hours of incubation and T-cell proliferation evaluated using scintillation counting.

Drug-specific TCC are highly selective and shown no cross reactivity.

A DDS-specific TCC



Cross- reactivity

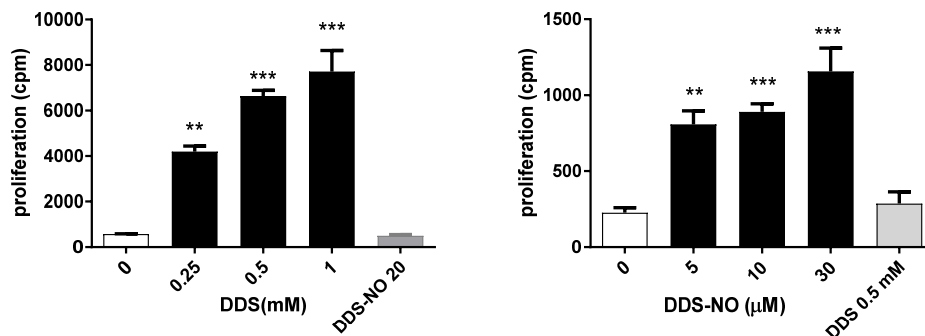


Figure 3.3: Dose response and cross reactivity of drug-specific TCC: A) DDS-specific TCCs were co-cultured with irradiated autologous EBV-transformed B cells in the presence of graded concentration of DDS (0.125-1mM) DDS-NO or SMX-NO. In a 96 wells plate at 5% CO₂ for 48 h. Proliferation was determined using radioactive thymidine incorporation during the final 16 hours of incubation and T cell proliferation evaluated using scintillation counting. B) DDS-NO-specific TCC was cultured with graded concentration of DDS-NO. Data was analysed in the same way as discussed above.

3.3.2 T-cell CD phenotyping

All the three DDS-specific TCC expressed CD4 cell surface protein. In contrast, the one DDS-NO-specific TCC that could be expanded sufficiently was CD8+. (Figure 3.4).

CD4/CD8 characterisation of drug-specific TCC.

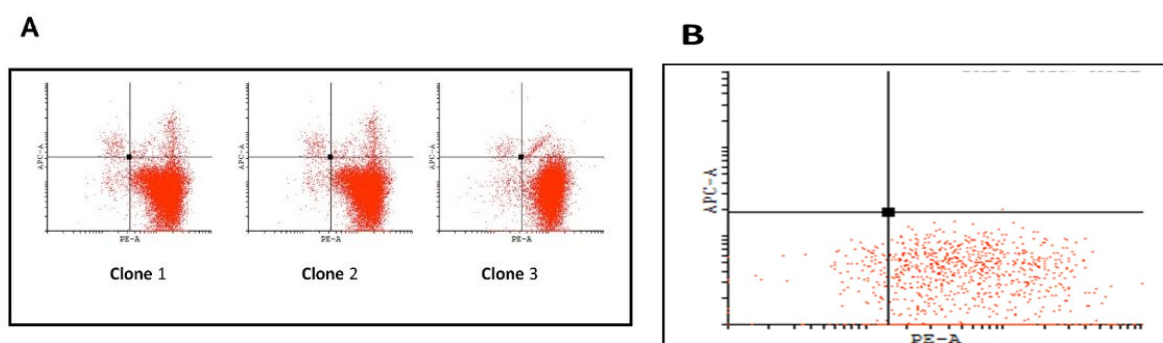


Figure 3.4:A) The phenotypes of three DDS-specific TCCs were determined using fluorescent activated cell sorting with fluorescent antibodies for CD4 (PE) and CD8 (APC). B) The phenotype of one DDS-NO-specific TCC was determined using fluorescent activated cell sorting with fluorescent antibodies for CD4 (APC) and CD8 (PE).

3.3.3 The effect of glutathione on the activation of DDS and DDS-NO specific TCC.

APC and TCC were pre-treated with glutathione (1 mM) before incubation with DDS (0.5 mM) or DDS-NO (10 μ M). Glutathione had no effect on the activation of DDS-specific clones, in the contrast to the T-lymphocyte proliferative responses of DDS-NO-specific clones, which was reduced in the presence of glutathione. (Figure 3.5).

Glutathione alternate the activation of DDS-NO-specific TCC.

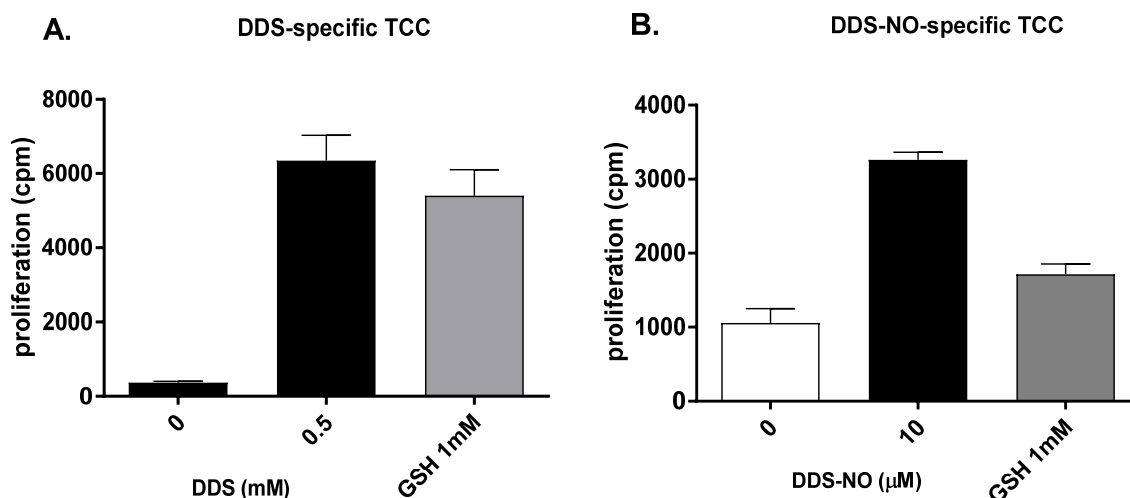


Figure 3.5: [^3H]-proliferation assay, DDS-specific TCC was co-cultured with irradiated autologous EBV-transformed B-cells and DDS in a 96 plate wells at 5% CO_2 for 48 h in the presence and absence of glutathione (1mM). Proliferation was determined using radioactive thymidine incorporation during the final 16 hours of incubation and T cell proliferation evaluated using scintillation counting. B.DDS-NO specific TCC was activated with APC and DDS-NO in the absence or presence of glutathione as describe above and proliferate response measured using [^3H] thymidine.

3.3.4 Characterisation of the mechanism of activation of DDS and DDS-NO-specific TCC.

The fixation of the APC is important to determine the role of antigen processing in T cell activation. Glutaraldehyde fixation of APC abolishes the ability of APC to process antigens presenting. DDS-specific T cell clones were stimulated via a processing independent pathway involving a direct interaction between DDS-MHC peptide complex and T cell receptors as clones were activated with the drug in the presence of fixed APC (Figure 3.6C). Similarly, DDS-specific T-cell clones were not activated with DDS pulsed APC. In contrast, DDS-NO pulsed APC activated the DDS-NO-specific clones (Figure 3.6E) and fixation of antigen presenting cell blocked the activation of clones with DDS-NO. CD4^+ DDS-specific TCC were MHC class II restricted (Figure 3.6A). Surprisingly, the DDS-NO-specific TCC was CD8^+ (Figure 3.4B) and MHC class II (Figure 3.6D).

Molecular mechanism of DDS-specific TCC activation.

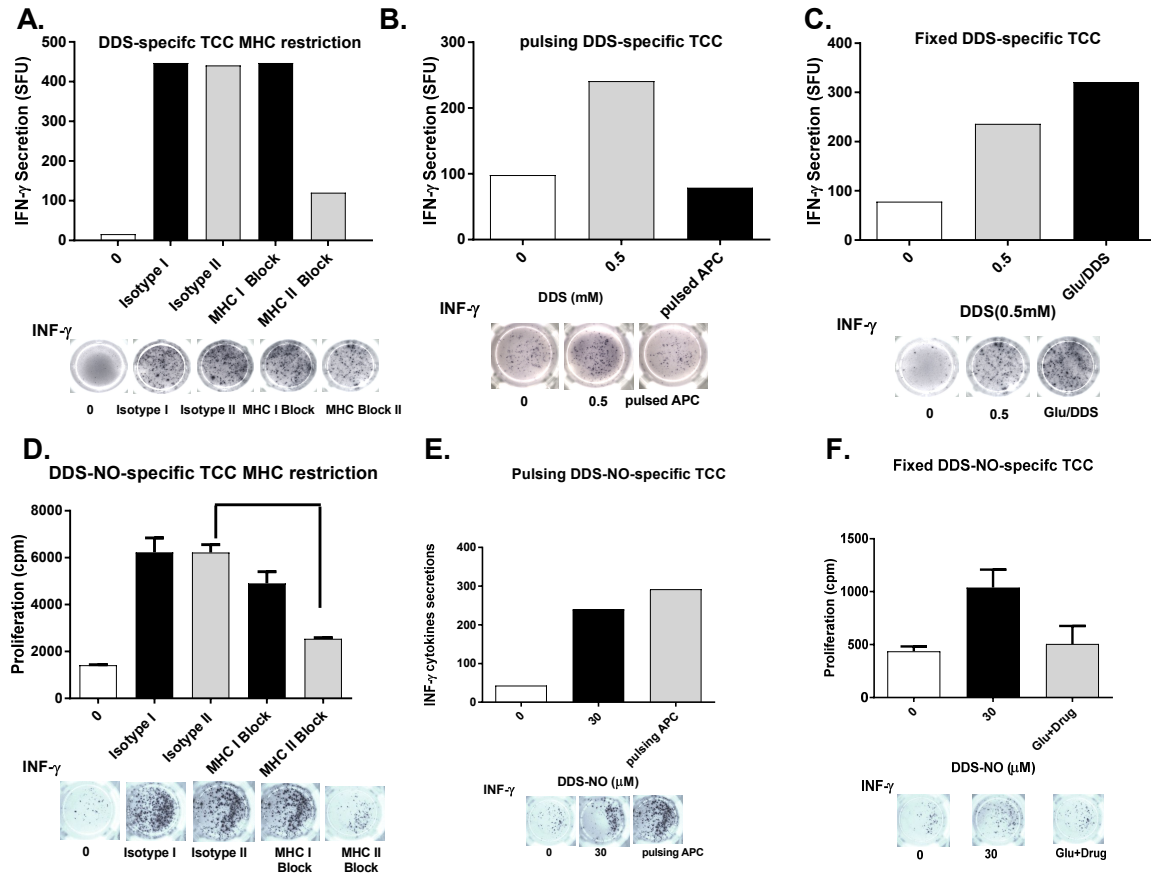


Figure 3.6 mechanism of activation T-cell: A. DDS-specific TCC generated from two drug-naïve donors. ELISpot plates were pre-coated with human IFN- γ coating antibodies according to manufacturer's instruction and incubated overnight at 4°C. TCCs were co-incubated with irradiated autologous EBV-transformed B-cells and DDS (0.125 -1 mM) using culture medium as negative control in the presence either MHC I or MHC II blocking antibodies. Isotype control were used as negative control the plates were incubated at 37°C, 5% CO₂ for 48 h and plates developed for image visualisation and spot counts. B. EBV-transformed B-cells were pulsed with DDS for 24 h and cells washed extensively to remove free drug. Drug-pulsed EBVs were then co-cultured with DDS-specific TCC, and IFN- γ secretion determined as described above. C. EBVs were first treated with glutaraldehyde to abolish antigen processing then co-cultured with DDS-specific TCC and DDS followed by IFN- γ ELISpot assay. In contrast, fixation of APC blocked the activation of DDS-NO-specific T-cell clones. D, E and F represent similar experiments described above for DDS-NO-specific TCC.

3.3.5 Drug-specific TCC secrete a mixture of Th1, Th2 and cytokines molecules.

The cytokines IL-13 and IFN- γ , were secreted from DDS or DDS-NO stimulated clones generated from drug-naïve healthy donors. (Figure 3.7) TCC failed to secreted either IL-5 or the cytolytic molecules granzyme B.

Cytokine secretion profiles of DDS-and DDS-NO-specific TCC.

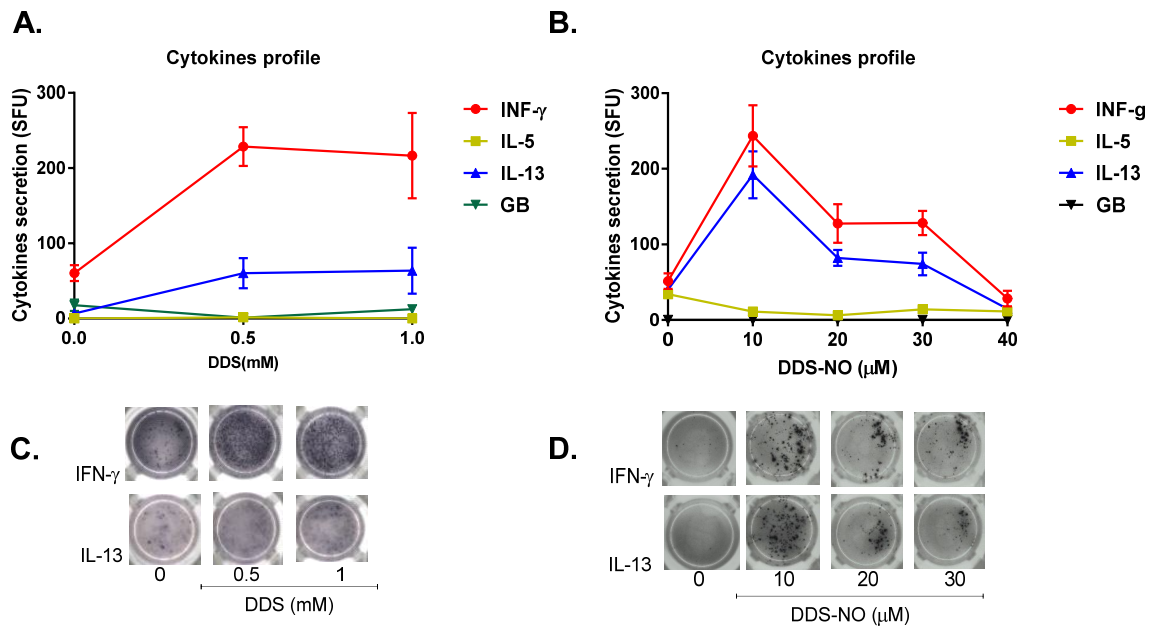


Figure 3.7: Cytokine secretion profiles of DDS-and DDS-NO-specific TCC generated from two drug-naïve donors. ELISpot plates were pre-coated with human INF- γ , IL-5, IL-13 and granzyme-B coating antibodies according to manufacturer's instruction and incubated overnight at 4°C. TCCs were co-incubated with irradiated autologous EBV-transformed B-cells and DDS (0.125 -1 mM) or D-NO (10-40 μ M) using culture medium as negative control. The plates were incubated at 37°C, 5% CO₂ for 48 h and plates developed for image visualisation and spot counts. A and C show representative cytokine profile and images for DDS-specific TCC while B and D show representative cytokine profile and images for DDS-NO-specific TCC.

3.3.6 DDS-specific TCC clone cross reactivity to analogues DDS or sulfonamides.

Activation of DDS-specific clones was not observed when they were cultured with APC and analogues: 4, 4 thiodianiline; (DDE), 4, 4 oxydianiline (DDT) and 3, 3 sulfonyldianiline (3DDS); (Figure 3.8) or sulfonamides (sulfamethoxazole, sulfamerazine, sulfadiazine, sulfachloropyridazine, sulfadoxin and sulphanilamide (Figure 3.9). However, a weak proliferative response and IFN- γ release was detected when the DDS-specific TCC were cultured with the mono- and di-acetylated forms of DDS (Figure 3.8A&B).

DDS-specific TCC do not cross react with closely related dapsone analogues.

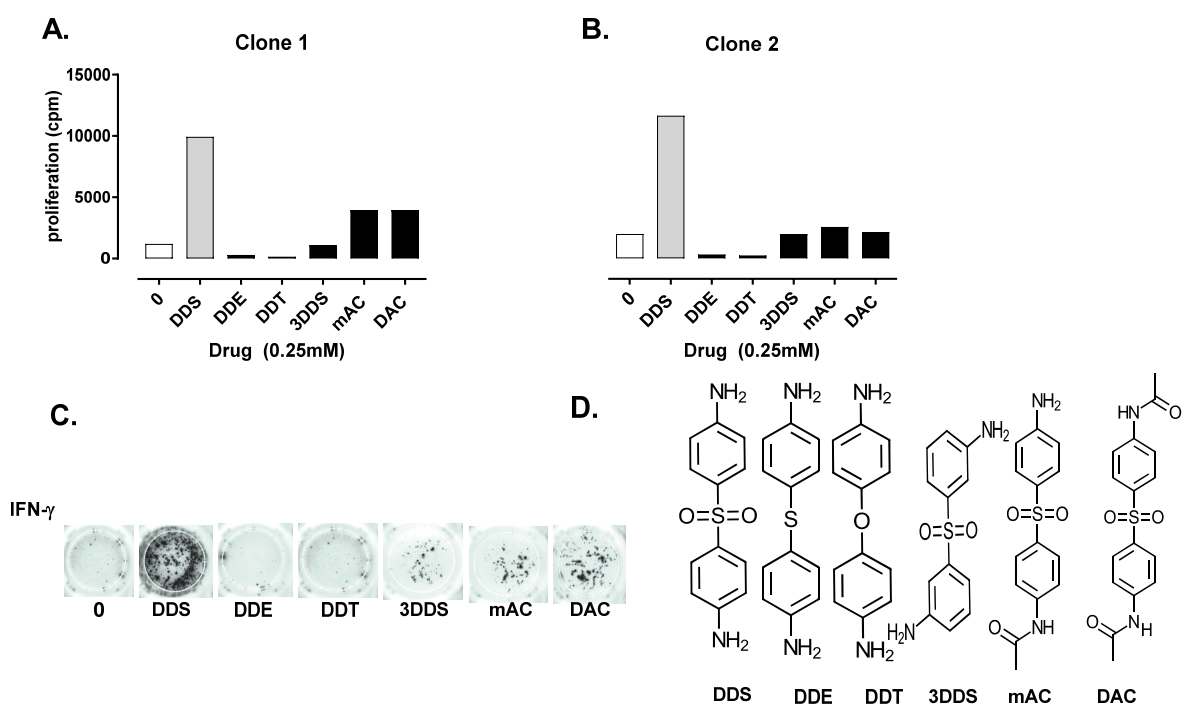


Figure 3.8: A, B) Cross reactivity of DDS-specific TCC with closely related analogues: DDS-specific TCC were co-incubated with irradiated autologous EBV-transformed B-cells in the presence of DDS and closely related analogues in a 96 wells plate at 5% CO₂ for 48 h. C) ELISpot images for DDS-specific TCC incubated with either DDS or closely related dapsone analogues (3DDS, DDT, DDE, mAC and DAC - 0.5 mM), D) DDS analogue chemical structures. Analogues (4,4 thiodianiline (DDE), 4,4 oxydianiline (DDT) and 3,3 sulfonyldianiline 3DDS, monoacetylate-dapsone (mAC), Diacetylate-dapsone (DAC).

DDS-specific TCC do not cross react with closely related sulfonamides.

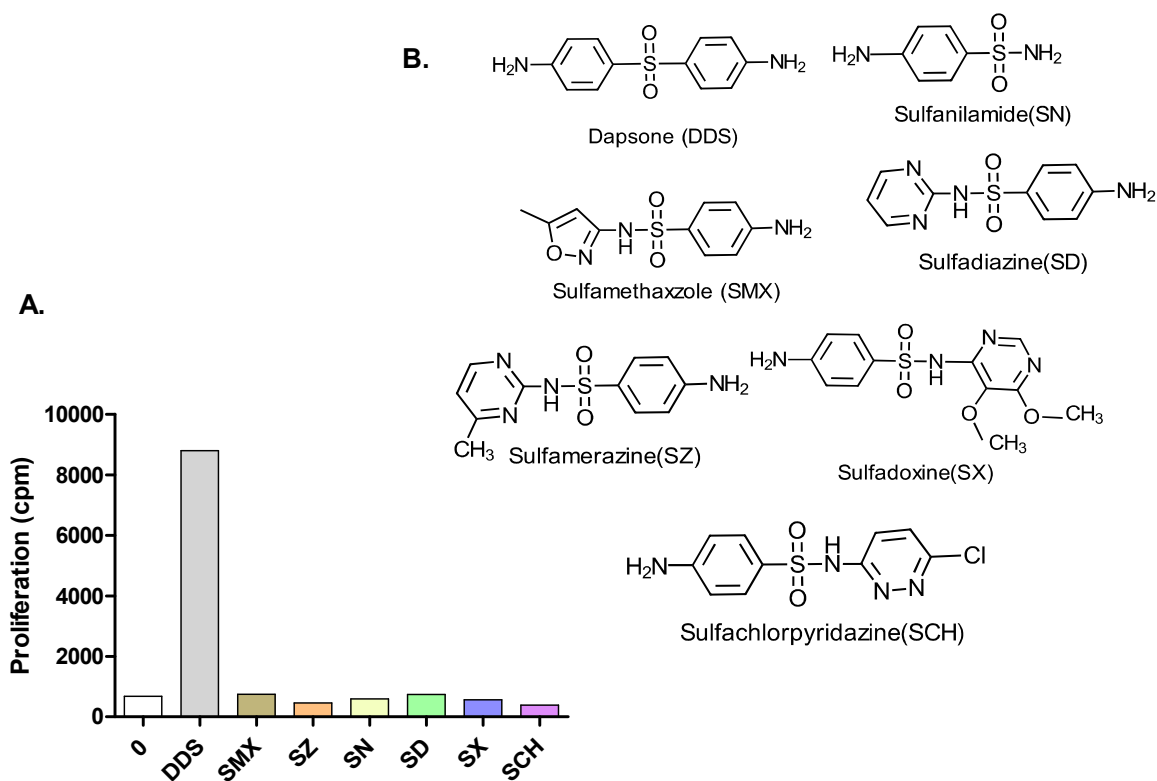


Figure 3.9: A) Cross reactivity of DDS-specific TCC with closely related sulfonamides : DDS-specific TCC was co-incubated with irradiated autologous EBV-transformed B-cells in the presence of DDS or different sulphonamides (SMX , SZ,SD , SN , SX ,SCH- 0.5 mM) in 96 wells plate at 5% CO₂ for 48 h. (B) Chemical structure of sulfonamide tested in cross reactivity by using thymidine proliferation assay.

3.4 Discussion

A clear understanding of the mechanistic basis of drug hypersensitivity remains a significant challenge as genetic, environmental and chemical factors impact upon susceptibility and the nature of the adverse event (Uetrecht & Naisbitt 2013). Layered on top of this is the fact that drugs interact with immune receptors in a number of ways to initiate an antigen-specific T-cell response. The dearth of synthetic protein-reactive metabolites for functional studies has complicated the development of research strategies to explore drug antigen presentation to T-cells. Thus, the majority of studies have focused on the interaction of parent drugs with MHC. For this reason, the aim of the current study was to explore the immunogenicity of the DDS and DDS-NO in human test systems. In this study dapsone was selected because of the dapsone hypersensitivity syndrome that develops in dapsone treated Chinese patients with leprosy, and its strong association with the expression of a single HLA allele, B*13:01 (Zhang et al. 2013).

DDS-NO was synthesized by oxidation of DDS hydroxylamine using the method established by Naisbitt *et al* (Naisbitt et al. 1996). To explore dapsone immunogenicity, we utilized a recently established assay (Faulkner et al. 2016; Faulkner et al. 2012) that involves the culture of dendritic cells with autologous naïve T-cells and either DDS or DDS-NO for 8 days. In chapter 2, naïve T-cells from healthy donors were primed to either DDS or DDS-NO. TCC were subsequently generated from two donors. DDS-specific TCC were CD4⁺, whereas CD8⁺ TCC were activated with DDS-NO. Importantly, the clones did not display cross-reactivity.

Ourselves and others have developed and utilised a battery of functional assays to distinguish between hapten and pharmacological pathways of T-cell activation (Meng et al. 2017; Schnyder et al. 2000; Castrejon et al. 2010; Monshi et al. 2013; Burkhart et al. 2001; Nassif et al. 2004). The same approach was adopted herein to define pathways of DDS- and DDS-NO-

specific activation of TCC. The activation of TCC with both DDS and DDS-NO was dependent on the presence of antigen presenting cells. Antigen presenting cells were therefore pulsed with DDS or DDS-NO followed by repeated washing to remove unbound drug, to explore whether TCC are activated by DDS (metabolite)-modified proteins. DDS-NO-specific TCC were activated with DDS-NO-pulsed but not DDS-pulsed antigen presenting cells. In contrast, DDS-specific TCC were not activated with antigen presenting cells pulsed with either DDS or DDS-NO. In the next experiments, glutathione was added to the T-cell proliferation assay. As discussed above, glutathione interacts covalently with DDS-NO limiting protein binding. Glutathione reduced the activation of TCC with DDS-NO, while it had no effect on the DDS-specific TCC. Collectively, these data indicate that DDS-NO forms a protein adduct within antigen presenting cells to activate TCC, whereas DDS-specific TCC are activated via a pharmacological interaction of the parent drug with MHC and the T-cell receptor.

Fixation of antigen presenting cells with glutaraldehyde blocks antigen processing, but not the display of MHC molecules on the cells surface (Zanni et al. 1998). To confirm that the processing of proteins into peptide fragments is involved in the activation of DDS-NO-, but not DDS-, specific TCC, glutaraldehyde-fixed antigen presenting cells were used in the proliferation assay. TCC were not activated with DDS-NO, whereas DDS activated TCC to a similar extent when experiments with fixed and irradiated antigen presenting cells were compared.

Sulfones such as DDS are used rarely in pharmacology, when compared with drug classes such as the sulfonamides. For this reason, we chose to explore the reactivity of DDS-specific TCC with DSS analogues and a panel of sulfonamides. TCC were not stimulated to proliferate with 4,4 thiodianiline, 4,4 oxydianiline or 3,3 sulfonyldianiline, which indicates that the sulfone moiety ($O=S=O$) and the position of the amine groups are important for T-cell activation.

Similarly, TCC were not activated with the panel of sulfonamides. Hence, the interaction of DDS with MHC that leads to the T-cell response seems to be highly structurally specific.

In addition to *N*-hydroxylation, which yields DDS hydroxylamine and DDS-NO, DDS is metabolized by *N*-acetyl transferase enzymes to mono- and di-acetylated forms (Zuidema et al. 1986), which are presumed to be non-toxic. Certain DDS-specific clones were stimulated to proliferate weakly and secrete low levels of IFN- γ in the presence of both mono- and di-acetylated DDS, suggesting that these species also interact with MHC molecules to stimulate T-cells. Further experiments however are needed to explore whether the acetylated DDS metabolites prime naïve T-cells.

In summary, our data indicate that DDS-NO can activate human naïve T-cells via a hapten pathway. In contrast, the parent drug binds to MHC molecules expressed on the surface of antigen presenting cells to activate T-cells. It is important to emphasize that our healthy donors did not express the HLA risk allele HLA-B*13:01. In chapter 4 we recruited HLA-B*13:01+ donors with a history of DDS hypersensitivity to explore the nature of the drug-specific T-cell response and whether DDS and/or DDS-NO bind preferentially to HLA-B*13:01 to selectively activate CD8⁺ T-cells.

Chapter 4: Generation and characterisation of DDS- and DDS-NO-specific T-cell from 6 HLA-B*13:01 positive patients.

Table of contents

4.1 Introduction:	110
4.2. Materials and Methods	113
4.2.1 Patients Characteristics.	113
4.2.2 T-cell cloning and generating autologous antigen presenting cell (APC).	114
4.2.3 Lymphocyte transformation test (LTT) for DHS patients.	115
4.2.4 Assessment of the specificity of TCC.	115
4.2.5 Antigen presenting cell (APC) fixation and antigen pulsing assays	115
4.2.6 MHC restriction assay.	116
4.2.7 T-cell V β receptor analysis.	116
4.3 Results:	117
4.3.1 Lymphocyte transformation test (LTT)	117
4.3.2 Secretion of IFN- γ from patient PBMC co-cultured with DDS or DDS-NO.	120
4.3.3 Generation of drug-specific T-cell clones.	121
4.3.4 T-cell phenotyping (Flow cytometry)	124
4.3.5 Drug-specific T-cell secrete a cytokines molecules.	125
4.3.6 Role of antigen presenting cells in the activation of TCC.	127
4.3.7 Cross reactivity of DDS-specific TCC with closely-related DDS analogues.	128
4.3.8 Cross reactivity DDS-specific TCC with sulfonamide antimicrobials.	129
4.3.9 Cross of TCC reactivity with DDS metabolites.	131
4.3.10 T-cell glutathione inhibited activation DDS and DDS-NO-specific TCC.	132
4.3.11 Molecular mechanism for activation of DDS and DDS-NO-specific TCC.	133
4.3.12 T-cell V β receptor analysis.	135
4.3.13 Chemokines receptor analysis.	136
4.4 Discussion	139

4.1 Introduction:

Drug-induced hypersensitivity is a major cause of anxiety to the patient, hospital and pharmaceutical companies. For the patient, it may cause concern that may delay with their healing period. In terms of hospital concerns, the challenge is to determine the kind of treatment and the prescription of effective medication without causing more suffering. The Pharmaceutical industry incur significant loss in income when drugs are withdrawn from clinical use due to hypersensitivity reactions. Considering that the estimated cost of inventing, developing and testing a new drug is about \$5 billion (Tohyama and Hashimoto, 2011) the loss of one drug may threaten the existence of the pharmaceutical company. In many nations, 15 to 20 % of their hospital budget is used in treating adverse drug reactions. Also in England, about £2 billion is spent annually in treating adverse drug reactions (Veeran, and Weiss, 2017).

DDS is an anti-inflammatory agent and a treatment for infections such as leprosy. The use of DDS can lead to hypersensitivity in up to 3% of patients. A mortality of 3% has also been reported for those who develop DDS-mediated adverse drug reaction (Cornelius et al., 2016). According to Pichler et al. (2017) early detection of drug hypersensitivity is important in order to prevent the development of life-threatening hypersensitivity syndrome. DDS is an aniline derivative, it is metabolized in the liver via *N*-hydroxylation and *N*-acetylation pathways. Cytochrome P-450 mediated *N*-hydroxylation of dapson releases a metabolite called DDS hydroxylamine which is thought to be precursor to the ultimate compound responsible for DDS hypersensitivity. DDS hydroxylamine is spontaneously oxidised to a protein-reactive nitroso derivative DDS-NO. The organ most frequently and prominently affected in drug hypersensitivity syndrome is skin, and generalized exanthema (maculopapular rash) is the main cutaneous reaction. It is characterized by an elevated spotted rash which appears one to three days after drug intake; it starts on the trunk and then the limbs. Adverse drug reactions may also cause hardening of the lymph node and exfoliative dermatitis (Macy et al. 2017). Steven-

Johnson's syndrome (SJS) and toxic epidermal necrolysis (TEN) are the most life-threatening forms of cutaneous drug hypersensitivity reaction. SJS start as maculopapular rash and proceeds to bullae, conjunctivitis, fever and fatigue. TEN is rare but symptomatically presents as SJS but causes detachment of a large portion of epidermis and has a mortality rate of 30%. DDS causes a hypersensitivity syndrome involving a triad of effects including fever, skin rash and eosinophilia often with systemic involvement such as liver or haematological injury. Reactions have an onset of 2-8 weeks after commencing DDS treatment which is longer than for other forms of drug hypersensitivity. This had led researchers to suggest the pathomechanism for DDS hypersensitivity may be different to others form of skin injury. Specifically, Picard et al (2010) suggested that the hypersensitivity syndrome may involve an anti-viral T-cell responses promoted by exposure to the culprit drug. Both innate and the adaptive immunity may be stimulated by drug metabolism and the formation of reactive metabolites, either by protein binding which lead to MHC-associated T-cell activation or through stress activated triggering of innate cells (Daly, 2013). Drugs are small molecules that should not provoke an adaptive immune reaction. As a result, for a drug-induced immune reaction to occur, drugs are believed to acts as prohaptens or haptens. These chemically responsive molecules then bind to large protein. Traditionally it is believed that sensitization of T-cells happens when the drug-protein complex binds to antigen presenting cell (APC) and are transported to the nearest draining lymphoid tissue for processing and then presentation to MHC (Yun et al. 2016). Naïve T-cells are then triggered to proliferate into mature T-cells. Drug-hapten specific T-cells are stimulated with drug-modified peptide derived from the original protein when they are bound to MHC molecules. In this respect, the previous chapter found that naïve T-cell from healthy donors are activated with DDS-NO via a pathway dependent on covalent binding of the metabolite to protein or a peptide embedded within MHC. Pichler et al showed that drugs also activated T-cells via a vastly different pathway involving

the direct non-covalent modification of MHC molecules (Pichler et al. 1997; Schnyder et al. 2000; Zanni et al. 1998). Although the binding interaction is labile, it is sufficiently strong to trigger T-cell receptors. In the previous chapter, we found that DDS also activates T-cells from healthy donors through a direct binding interaction with antigen presenting cells.

Zhang et (2013) indicated that HLA-B*13:01 was a risk factor for dapsone hypersensitivity syndrome, the allele was observed in 86% of Chinese case-patients. Understanding the pathomechanism associated with these HLA alleles and may help to identify the initial steps in the hypersensitivity reaction. The most recent study using computational modelling suggests that the chemical structure of dapsone interacts selectively with the antigen-recognition site of HLA-B*13:01 (Watanabe et al. 2017). Therefore the dapsone might interact directly with HLA-B*13:01 in patients, and trigger T-cell responses. Despite this, to date the activation of hypersensitive patients T-cells with DDS or DDS metabolites has not been studied. Thus, in this chapter we obtained PBMC from 6 Chinese hypersensitive patients and studied T-cell responses to either DDS or DDS-NO. Furthermore, we investigated the molecular mechanism of DDS-induced T-cell activation.

4.2. Materials and Methods

Table 4.1: Summarizes the patients' demographics

Patients ID	Gender	Age (years)	Medication history	Onset of symptoms (days)	Clinical presentation	Skin patch test
1	Female	43	Dapsone, rifampin, and clofazimine	3	Fever, rash, and abnormal liver function tests	-
3	Female	25	Dapsone, rifampin, and clofazimine	28	Fever, rash, and abnormal liver function tests	-
5	Male	39	Dapsone, rifampin, and clofazimine	30	Fever	+
6	Male	41	Dapsone, rifampin, and clofazimine.	48	Fever, rash, and lymphadenopathy	+
7	Male	54	Dapsone, rifampin, and clofazimine	16	Fever, rash, and abnormal liver function tests	-
8	Female	27	Dapsone, rifampin, and clofazimine	17	Fever and abnormal liver function tests	+

4.2.1 Patients Characteristics.

PBMC were isolated from six dapsone hypersensitive Chinese patients. Table 1 summarizes the patients' demographics, medical history and clinical features of the hypersensitivity reactions. Approval for the study was acquired from the local research ethics committee in China and material transfer agreement was in place for cell transportation; informed written consent was obtained from each patient.

4.2.2 T-cell cloning and generating autologous antigen presenting cells (APC).

PBMC (1×10^6 /ml) from 6 hypersensitive patients were cultured with either DDS (0.5 mM) or DDS-NO (20 μ M). Culture medium was supplemented with IL-2 (200 IU/ml) on day 6 and 9 in order to expand the number of antigen-specific T-cells prior to cloning by serial dilutions on day 14. T-cells were plated into 96 well plates at 0.3, 1 and 3 cells per well and expanded with PHA using the protocol described in chapter 3. Epstein-Barr virus (EBV) transformed B-cell lines were generated by transformation of freshly isolated PBMC using supernatant from the virus-producing cell line B9.58 as described in chapter 3. Autologous Epstein-Barr virus (EBV)-transformed B-cell lines were used as APC in assays involving TCCs. Antigen-specificity was assessed by culturing irradiated EBV-transformed B cells (1×10^4 /well) and DDS or DDS-NO with drug-specific TCC (5×10^4 /well; 200 μ l) for 48 hours. Proliferation was measured by the addition of [3 H]-thymidine followed by scintillation counting. Clones with a stimulation index (proliferation due to drug / proliferation due to medium) greater than 2 were expanded by repetitive stimulation with irradiated allogeneic PBMC (5×10^5 /well), IL-2 (5 μ g/ml) and PHA (10 μ g/ml).

4.2.3 Lymphocyte transformation test (LTT) for DHS patients.

LTT was performed using PBMC isolated from the 6 patients using an established protocol (Nyfeler and Pichler 1997). Briefly, PBMC (1.5×10^5 cells, 100 μ L) were cultured with either 100 μ L DDS (0.125-0.5 mM) or DDS-NO (10-40 μ M) in triplicate wells in a 96-well U-bottom plate and incubated at 37°C under an atmosphere 5% CO₂ for 5 days. [³H]-thymidine (0.5 μ Ci/well) was added for the final 16 hours of incubation and lymphocyte proliferation was assessed as counts per minute (cpm) using liquid scintillation counter (Wallac microbeta trilux, PerkinElmer, Cambridge, UK).

4.2.4 Assessment of the specificity of TCC.

Dose-dependent proliferative responses to DDS-NO (5-20 μ M) and DDS (0.125-0.5 mM) and the profile of secreted cytokines (IFN- γ , IL-5, IL-13 and granzyme-B) were then measured by culturing TCC and irradiated APC with the drug. Proliferation and cytokine release were measured using [³H]-thymidine incorporation and ELISpot respectively. Cell phenotyping was performed by flow cytometry using CD4-FITC, CD8-PE antibodies. Twenty-four well transwell chambers with 5- μ m pores were used to measure chemotaxis. T-cells (0.1×10^5 ; n=10 clones) were placed in the upper chambers. CCL17/CCL27 (ligand for CCR4 and CCR10 respectively) was placed in the lower wells and the cells were incubated for 0.5-24 hours. Cells migrating to the lower chamber were counted using a hemocytometer.

4.2.5 Antigen presenting cell (APC) fixation and antigen pulsing assays

APC fixation was used to investigate the role of APC metabolism in the activation of TCC. For this, autologous EBV-transformed B-cells (2×10^6 cells/ml) were washed twice in HBSS to exclude FBS and resuspended in HBSS (1 ml). Next, glutaraldehyde (25%, 1 μ L) was added and then the cells were lightly mixed for 30 seconds. After this step, glycine (1ml of 1 M) was rapidly added to the mixture and the cells were mixed for a further 45 seconds. To remove glutaraldehyde, cells were washed three times and were then resuspended in T cell culture medium. T cell clones

(5×10^4 , 50 μ L) were co-cultured with glutaraldehyde-fixed EBV-transformed B-cells (1×10^4 cells, 50 μ L) in the presence or absence of DDS (0.5 mM). In other experiments, APC - pulsed with DDS or DDS-NO for 16 hours were washed extensively with HBSS to exclude free drug and co-incubated with TCC in a 96-well plate for 48 hours under 5% CO₂ at 37°C. [³H]-thymidine was added during the last 16 hours incubation and antigen specificity evaluated.

4.2.6 MHC restriction assay

In order to define whether DDS or DDS-NO presentation to TCC was MHC class I/II restricted, anti-human MHC I (HLA-A, -B, -C), and anti-human MHC II (HLA-DP, -DQ, -DR) antibodies (5 μ g/mL) were used. In the initiation of the experiment, autologous EBV-transformed B-cell lines (1×10^4 , 50 μ L) were pre-incubated with either MHC I or MHC II blocking anti-bodies (5 μ g/ml) at 5% CO₂, 37°C for 30 min. The APCs were then co-cultured with TCC (5×10^4 , 50 μ L) with or without drug for 48 hours. [³H]-thymidine (0.5 μ Ci) was added for the final 16 hours of incubation and T cell proliferation evaluated using scintillation counting.

4.2.7 T-cell V β receptor analysis.

Ten tubes (1-10) were required for TCR V β typing of individual clones. T-cell suspensions (50 μ L) were pipetted into each tube. Anti-CD3 antibody (3 μ L) was introduced into tubes 2-10. TCR V β antibodies (5 μ L) labelled A-H were then introduced into tubes 3-10 containing TCCs + anti CD3 antibody. Each TCR V β antibody cocktail was used to investigate three TCRs. Tube 1 had no antibody and was used to gate the T-lymphocyte population during flow cytometry. Tubes were incubated at room temperature for 20 minutes. Unbound antibodies were washed with FACS buffer (1 ml, 1500 rpm for 5 minutes at room temperature). Finally, TCCs were resuspended in FACS buffer (200 μ L) and samples analysed.

4.3 Results:

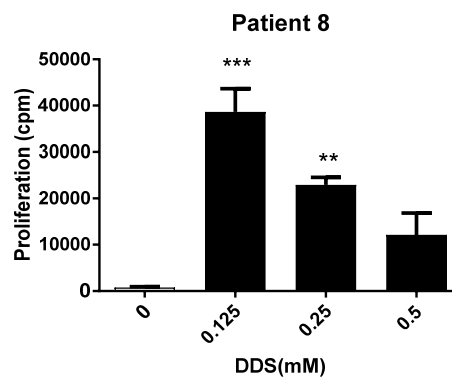
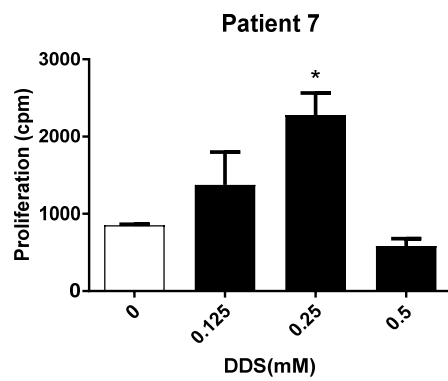
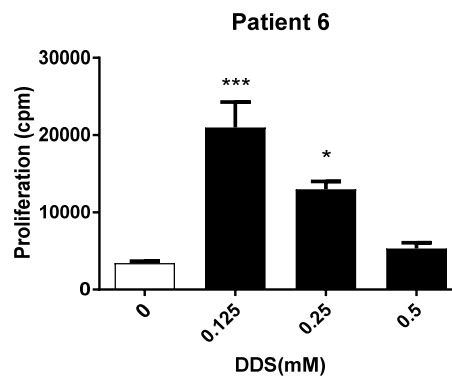
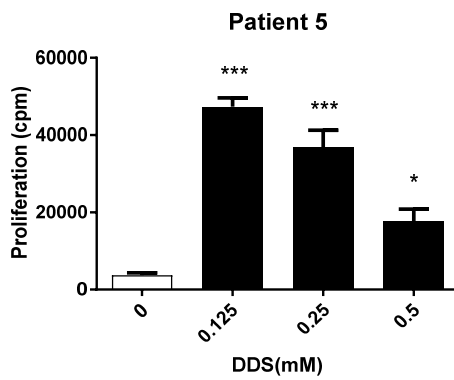
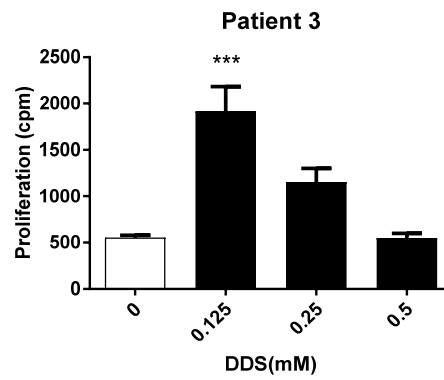
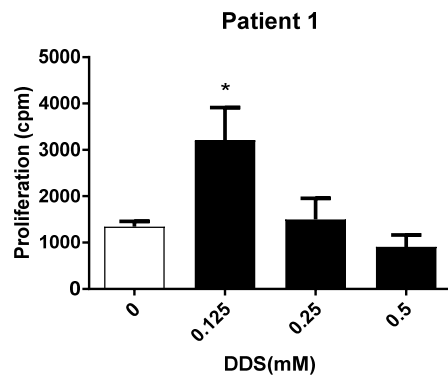
4.3.1 Lymphocyte transformation test (LTT)

We obtained blood samples from 6 patients with DDS hypersensitivity syndrome all of whom were HLA-B 13:01 positive PBMC were isolated and proliferative responses to DDS and DDS-NO were evaluated. Lymphocyte proliferation to graded concentrations of DDS and DDS-NO was observed. The proliferative responses were observed with PBMC from 6 patients in the presence of DDS and 4 patients with DDS-NO. TT (tetanus toxin) was used as a positive control. (Table 4.2, Figure4.1).

Table 4.2: Summary of the patients' demographics and LTT and IFN- γ results.

PBMCs sample	Gender	Age	B*13:01	DHS	LTT results DDS	LTT results DDS-NO	IFN- γ Results DDS	IFN- γ Results DDS-NO
Patient 1	Female	43	+	+	(+)	(-)	(+)	(+)
Patient 3	Female	25	+	+	(+)	(+)	(+)	(+)
Patient 5	male	39	+	+	(+)	(+)	(-)	(-)
Patient 6	male	41	+	+	(+)	(+)	(-)	(-)
Patient 7	male	54	+	+	(+)	(-)	(-)	(-)
Patient 8	female	27	+	+	(+)	(+)	(+)	(+)

A. LTT to DDS



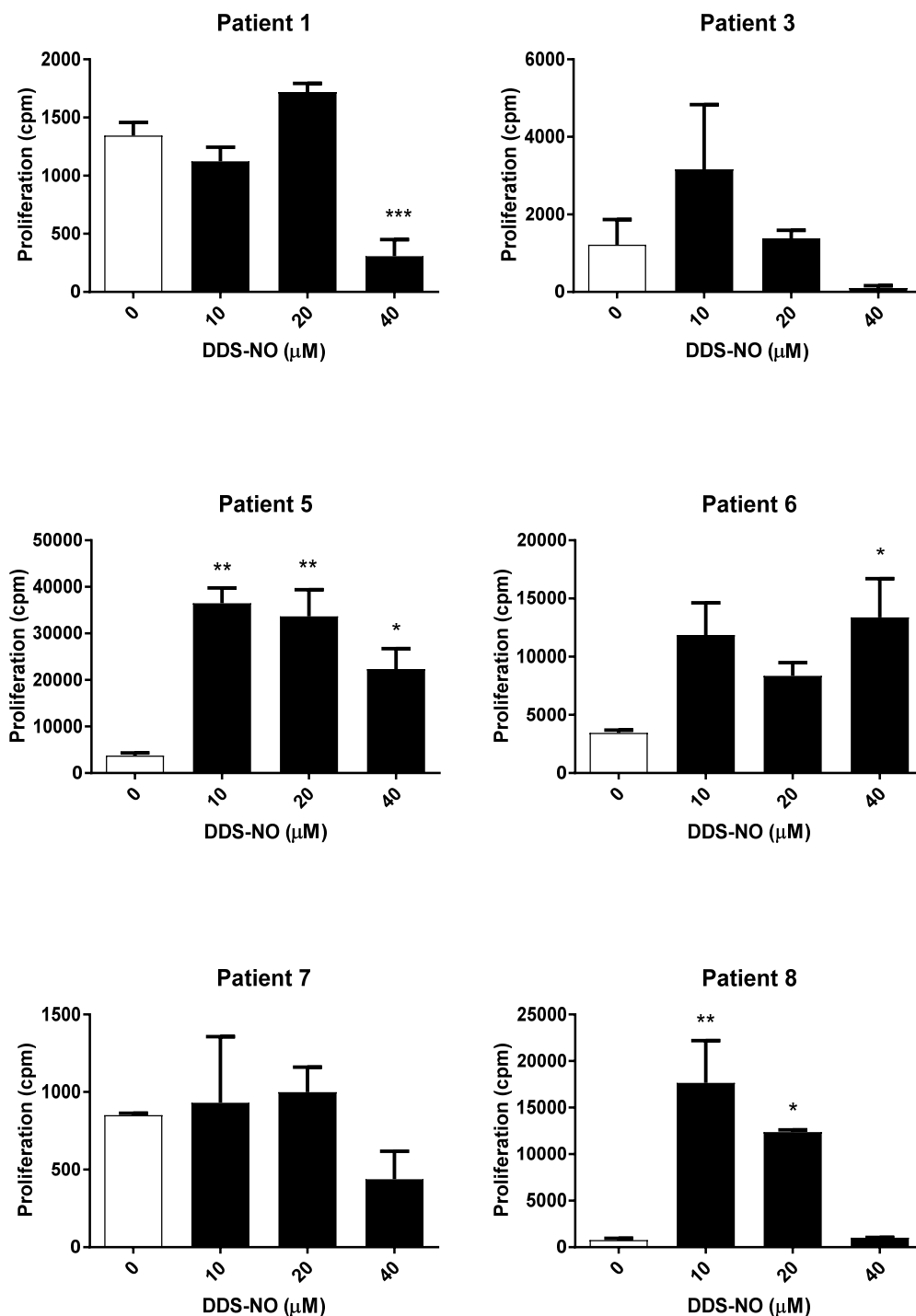
B. LTT to DDS-NO

Figure 4.1: Lymphocyte transformation test (LTT) assay. PBMC (1.5×10^4 cells, 100 μL) were incubated with graded concentrations of either (A) DDS (0.125-2 mM) or (B) DDS-NO (10-40 μM) in 96- well U-bottom plates. Plates were incubated at 37°C, 5% CO_2 for 5 days. [^3H]-thymidine (0.5 μCi) was added for the final 16 hours of incubation and T-cell proliferation evaluated using scintillation counting.

4.3.2 Secretion of IFN- γ from patient PBMC co-cultured with DDS or DDS-NO.

PBMC from six patients were cultured with DDS or DDS-NO and IFN- γ was measured by ELISpot. PBMC from 3 patients were stimulated to secrete IFN- γ with DDS and DDS-NO. Representative images with PBMC from responsive and non-responsive donors as shown in (Figure 4.2).

IFN- γ ELISpot for Dapsone-induced DHS patients

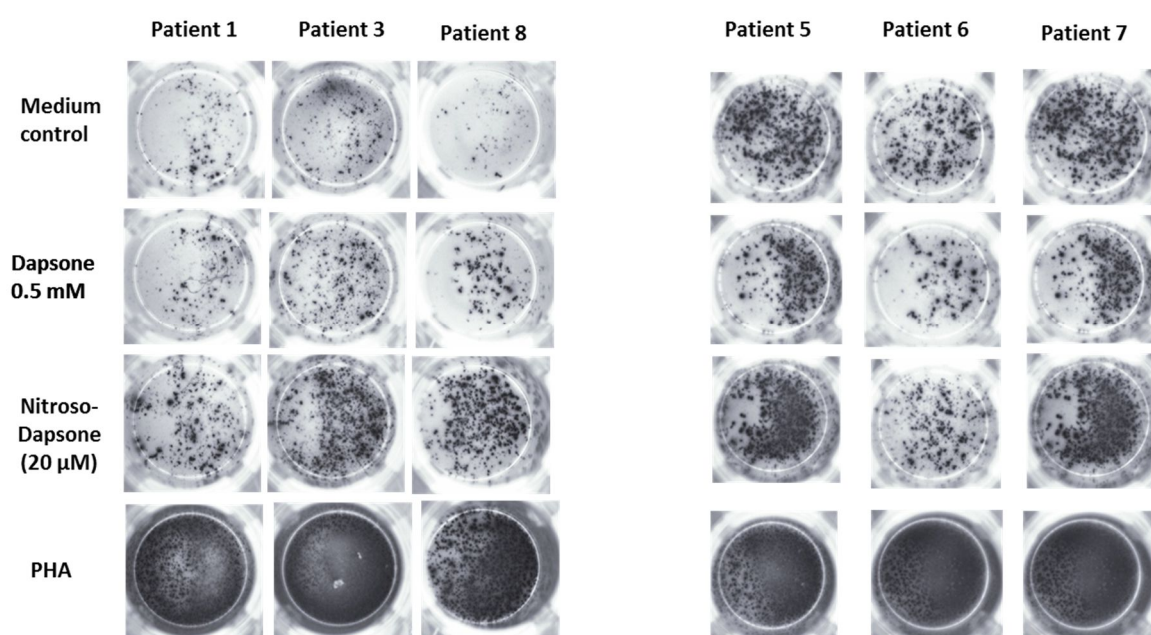


Figure 4.2: ELISpot plates were pre-coated with human IFN- γ coating antibodies according to manufacturer's instruction and incubated overnight at 4°C. PBMC (4×10^6 cells, 100 μ L) were co-incubated with DDS (0.5 mM, 100 μ L) or DDS-NO (20 μ M, 100 μ L) using culture medium as negative control and PHA as positive control. The plates were incubated at 37°C, 5% CO₂ for 48 h and plates developed for image visualisation and spot counts.

4.3.3 Generation of drug-specific T-cell clones.

Forty T-cell clones were generated from one DDS hypersensitive patient (P8, Figure 4.3). Of these, 12 showed a DDS-specific dose-dependent response. Cross reactivity with DDS-NO and DDShydroxylamine was observed with 50% of the clones. Out of 200 clones tested from 2 patients only two clones were DDS-NO responsive (P1, P8 Figure 4.3). These DDS-NO-specific cell clones showed a dose-dependent response to DDS-NO without cross reactivity to SMX-NO or DDS. None of clones were activated with SMX-NO (Figure 4.4).

Generation of dapsone and nitroso- dapsone-specific TCCs.

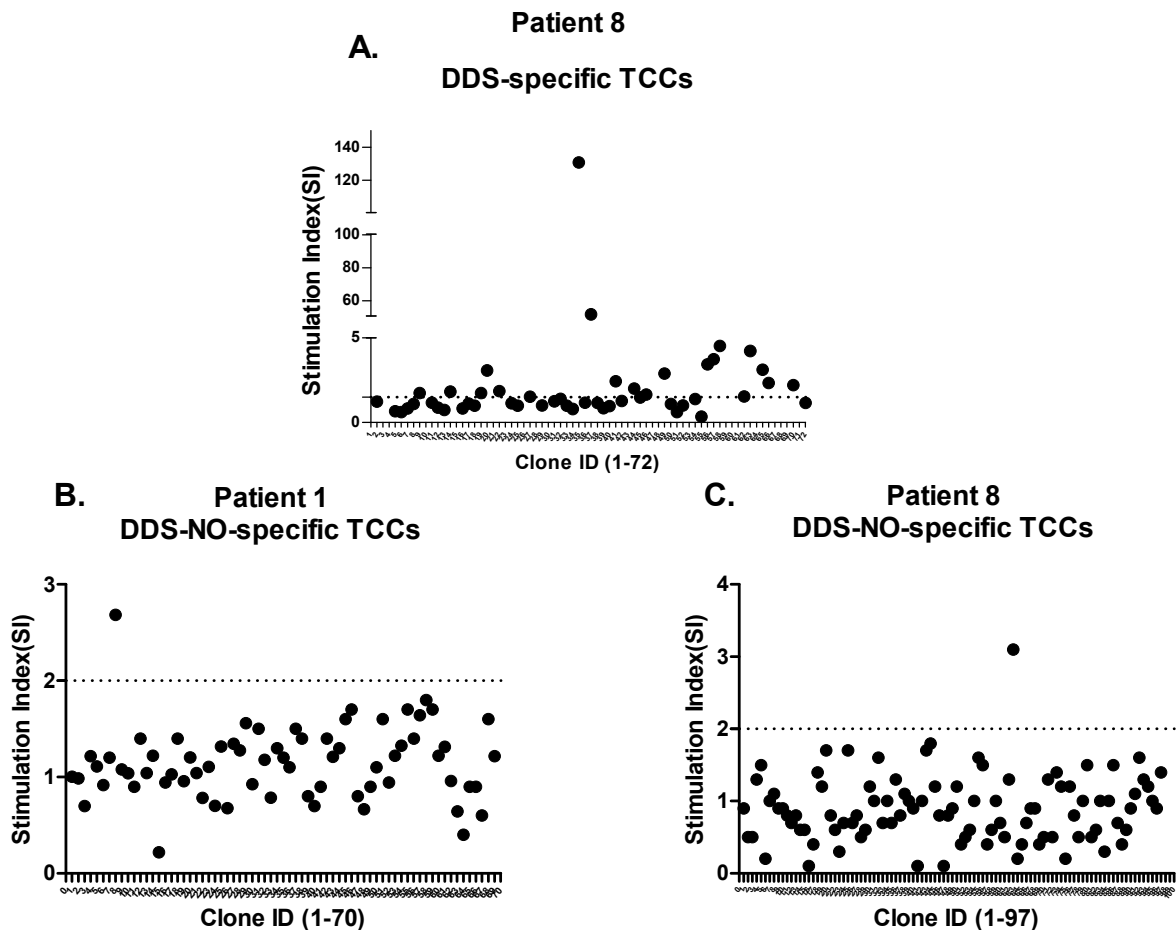
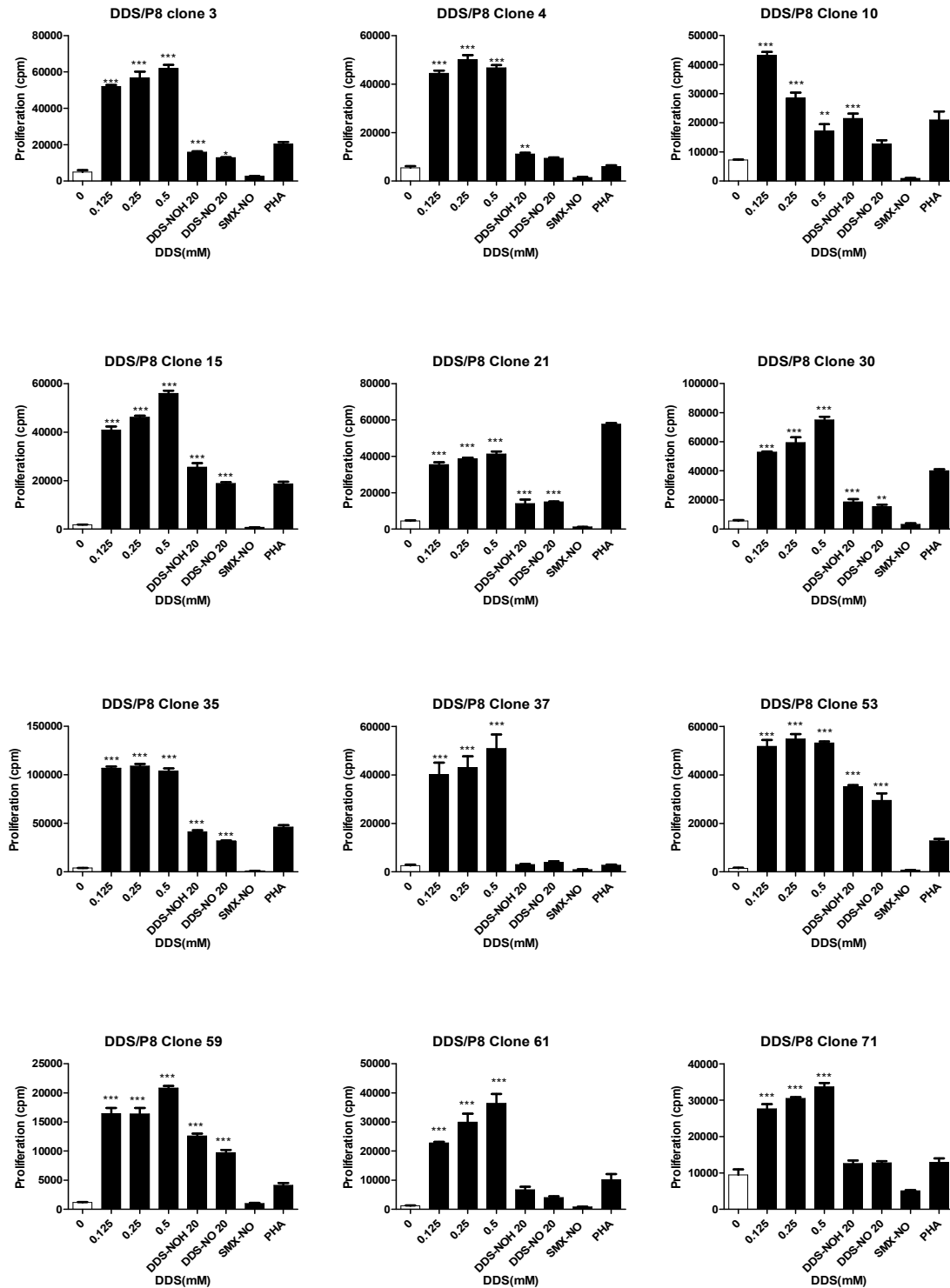


Figure 4.3: Generation of DDS-specific TCCs from one hypersensitive patients and DDS-NO-specific TCCs from two patients: TCCs were generated using serial dilution method. Clones were co-incubated with irradiated autologous EBV-transformed B cells and DDS or DDS-NO. Culture medium was utilised as negative control. The plates were incubated at 37 °C, 5%CO₂ for 48 h. Radioactive thymidine was added during the last 16 h of incubation. Clones with stimulation index > 2 were selected and expanded for further functional assays. A) DDS and B,C) DDS-NO TCCs generated from 2 patients.

Dose dependant activation of TCC and assessment of cross reactivity.

A. DDS-specific TCCs patient (8)



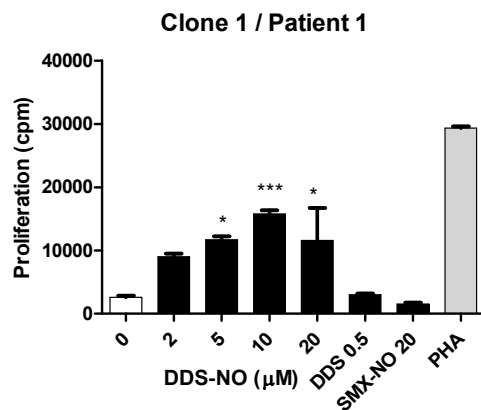
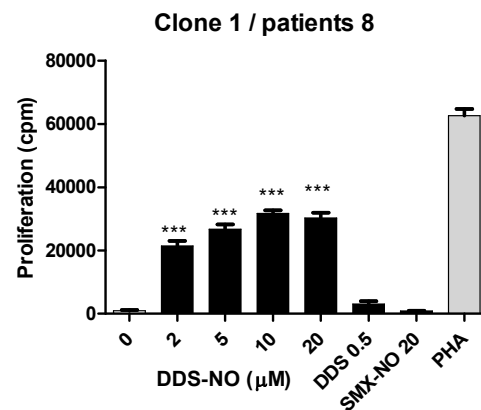
B. DDS-NO-specific TCCs patient 1**C. DDS-NO-specific TCCs patient 8**

Figure 4.4: Dose response and cross reactivity of drug-specific TCCs clones: A) DDS-specific TCCs were co-cultured with irradiated autologous EBV-transformed B cells and in the presence of graded concentration of DDS (0.125-1mM), DDS-NO or SMX-NO in a 96 wells plate at 5% CO₂ for 48 h. Proliferation was determined using radioactive thymidine incorporation during the final 16 hours of incubation and T cell proliferation evaluated using scintillation counting. B, C) DDS-NO-specific TCCs were cultured with graded concentration of DDS-NO. Data was analysed in the same way as discussed above.

4.3.4 T-cell phenotyping (Flow cytometry)

To determine the CD4 and CD8 phenotype of TCCs, flow cytometry was used. T cell suspensions (100 μ L) were stained with CD4-PE (3 μ L) and CD8-APC (3 μ L) antibodies and incubated at 4°C for 20 minutes in the dark. Cells were washed and resuspended in 200 μ L of FACS buffer and CD4, CD8 expression determined using the FACS Canto™ II system. The data shows that both DDS and DDS-NO specific clones express the CD4+ (Figure 4.5)

CD4/CD8 characterisation of drug-specific TCC.

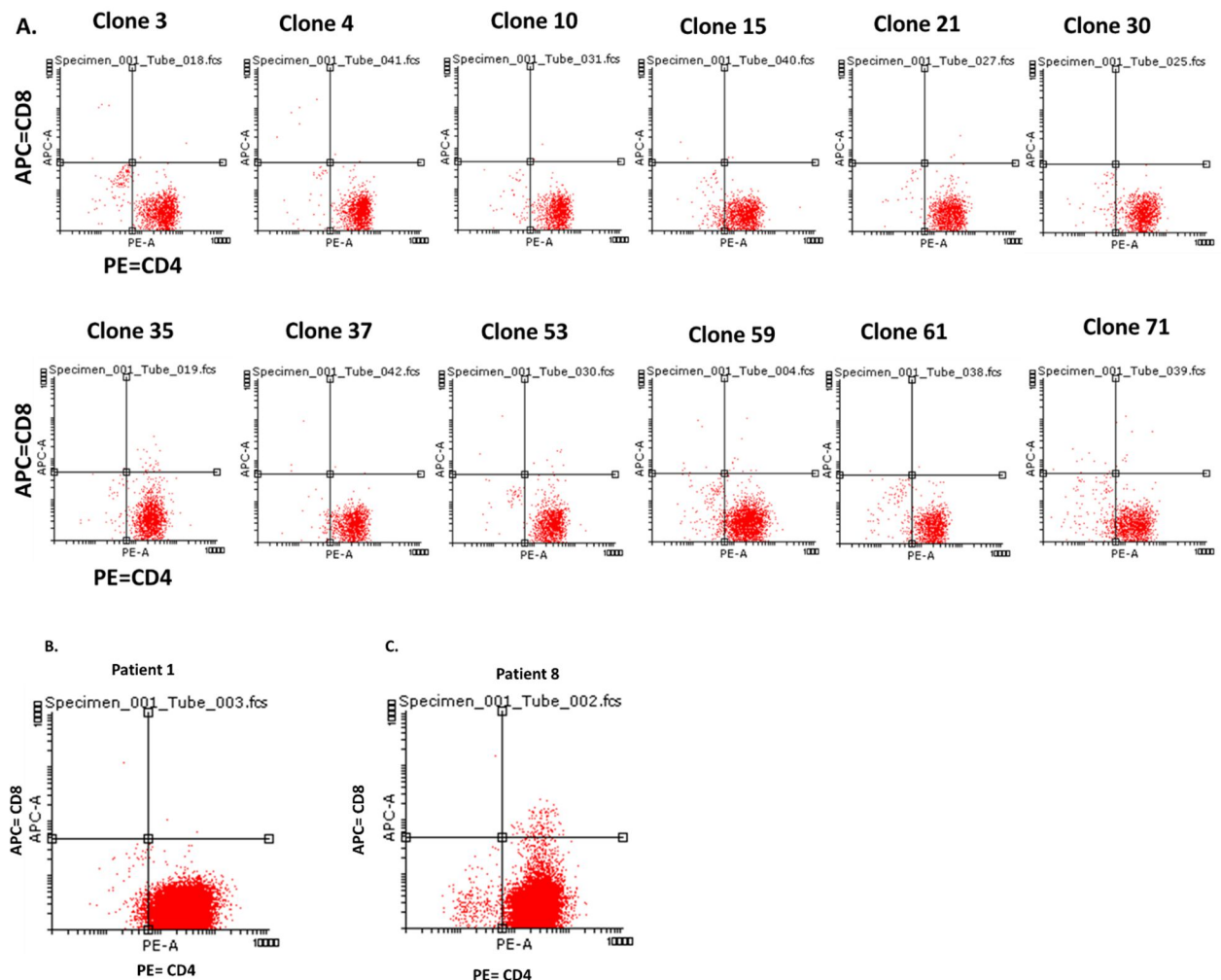
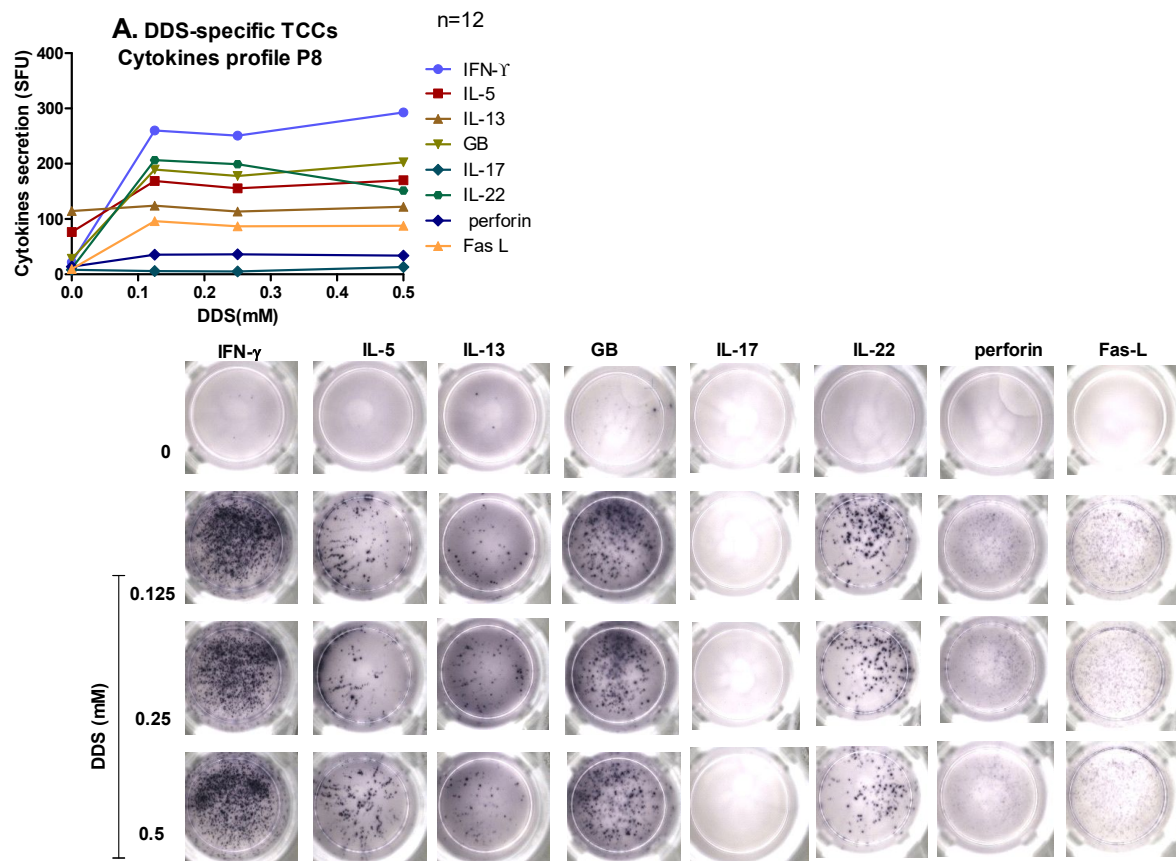


Figure 4.5: A. The phenotype of DDS-specific TCCs were determined using fluorescent activated cell sorting with fluorescent antibodies for CD4 (PE) and CD8 (APC). B,C. The phenotype of DDS-NO-specific TCCs was determined using fluorescent activated cell sorting with fluorescent antibodies for CD8(APC) and CD4(PE).

4.3.5 Drug-specific T-cells secrete cytokine molecules.

The cytokines IFN- γ , IL-5, IL-13, GB, IL-22, perforin and Fas-L, were secreted from DDS stimulated clones (Figure 4.6A). In contrast, drug-specific IL-17 secretion was not detected. The 1 DDS-NO responsive clones assessed secreted a similar panel of cytokines (Figure 4.6B). Interestingly, one clone also secreted IL-17 after stimulation with DDS-NO and autologous antigen presenting cells.

Cytokine secretion profiles of DDS-and DDS-NO-specific TCC.



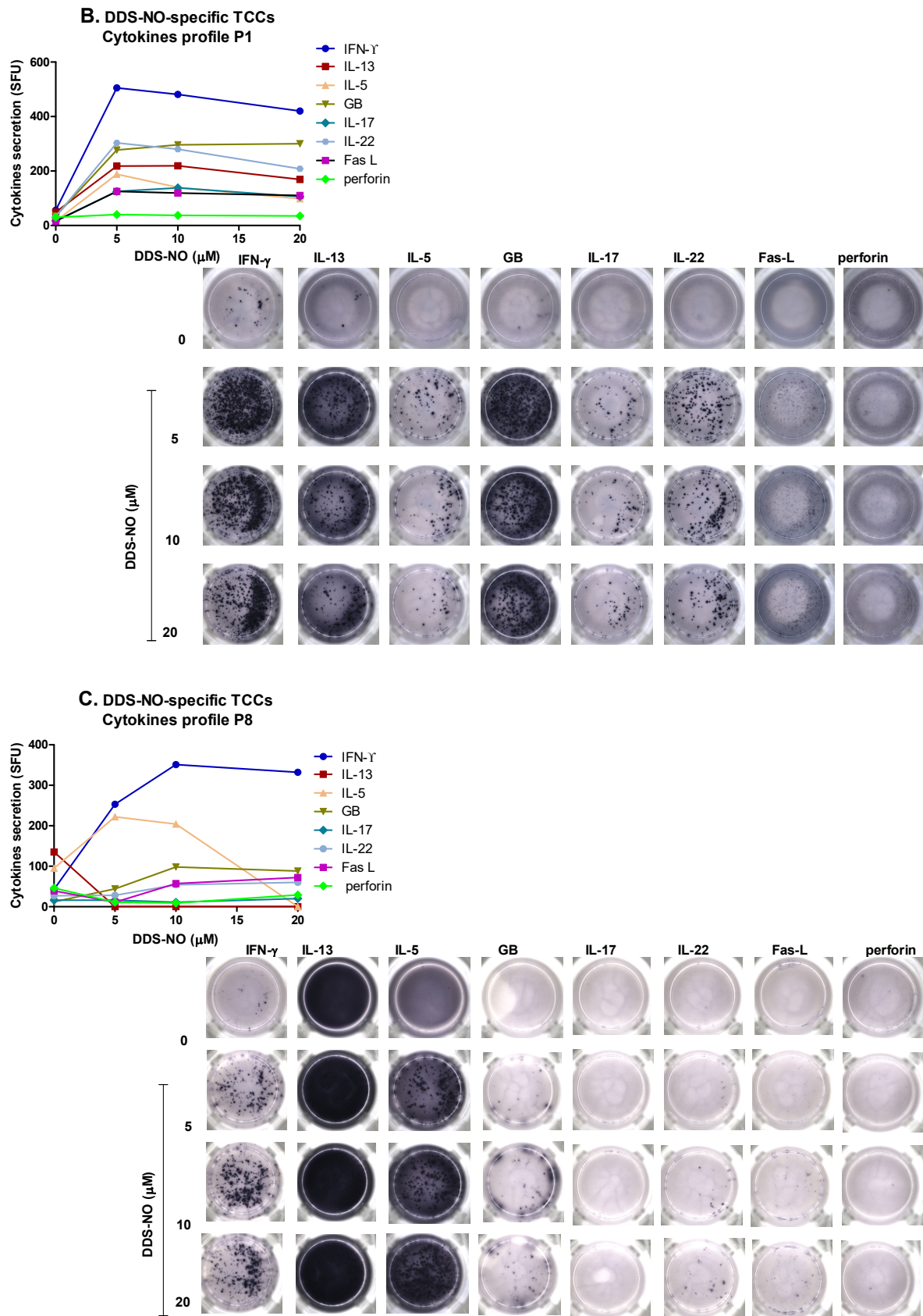


Figure 4.6: Cytokine secretion profiles of DDS- and DDS-NO-specific TCC generated from one and two hypersensitive patients respectively. ELISpot plates were pre-coated with human IFN- γ , IL-5, IL-13 and granzyme-B coating antibodies according to manufacturer's instruction and incubated overnight at 4°C. TCC were

co-incubated with irradiated autologous EBV-transformed B-cells and DDS (0.125-0.5 mM) or DDS-NO (5-20 μ M) using culture medium as negative control. The plates were incubated at 37°C, 5% CO₂ for 48 h and plates developed for image visualisation and spot counts. A represent cytokine profile and images for DDS-specific TCC while B and C show cytokine profiles and images for DDS-NO-specific TCCs.

4.3.6 Role of antigen presenting cells in the activation of TCC.

Experiments were performed by incubating DDS-specific or DDS-NO-specific TCC with or without autologous APC to prove the role of HLA alleles in T-cell activation. Fast growing DDS-specific TCC generated from Patient 1 (P8) and DDS-NO-specific TCC from Patient P1&P8 were used for the APC assay. All DDS and DDS-NO-specific clones were stimulated to proliferate with drug in the presence but not in absence of APC (Figure 7).

Antigen presenting cells are essential for the activation of DDS and DDS-NO-specific TCC.

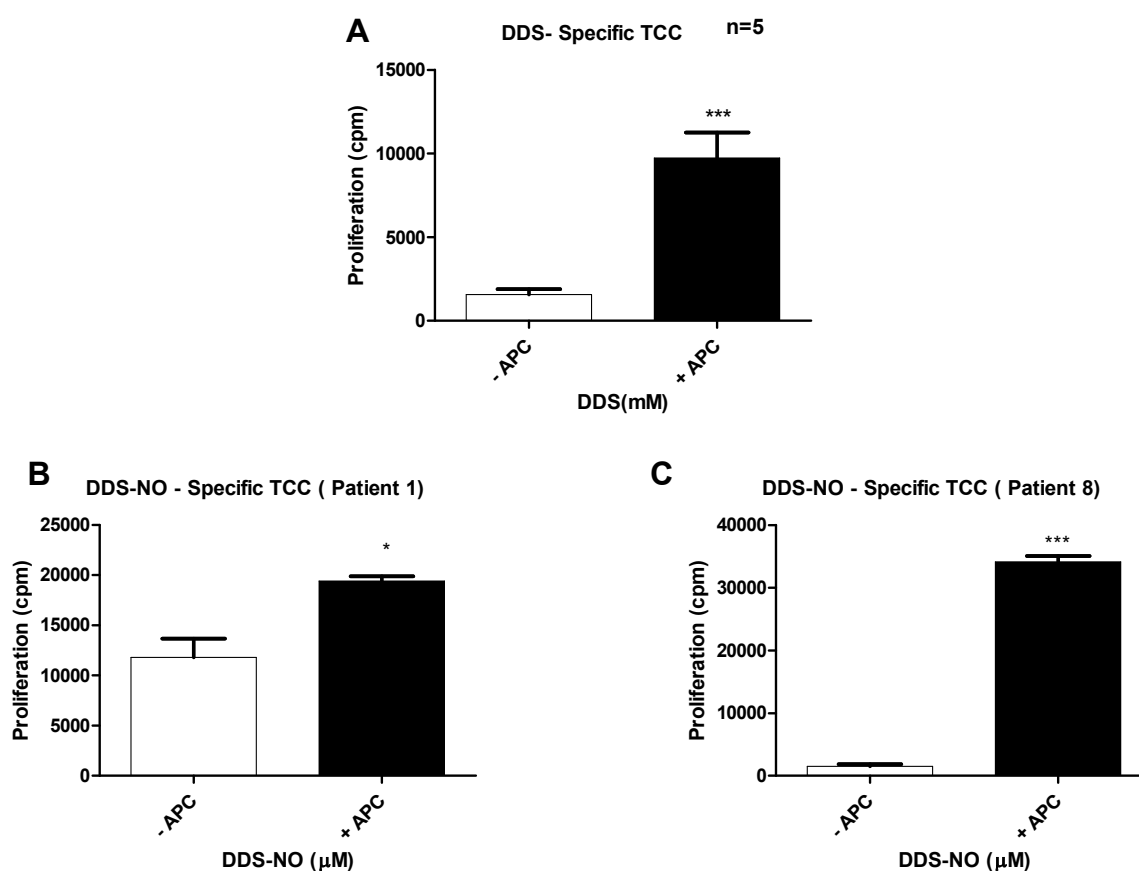


Figure 4.7: APC are important for the activation of A) DDS-specific TCCs and B, C) DDS-NO-specific TCC were co-cultured with or without irradiated autologous EBV-transformed B-cells and DDS and DDS-NO in a 96 wells plate and incubated at 5% CO₂ for 48 h. Proliferation was determined using radioactive thymidine incorporation during the final 16 hours of incubation and T-cell proliferation evaluated using scintillation counting.

4.3.7 Cross reactivity of DDS-specific TCC with closely-related DDS analogues.

Twelve clones generated from PBMC DDS-hypersensitive patients were tested for reactivity with closely related compounds. 2/12 TCC tested revealed a high proliferative responses with DDS and with ₃DDS (Figure 4.8). Both DDS and ₃DDS have aniline chemical groups at different positions on the aromatic rings. In contrast only one DDS-specific clone was activated with DDT at low concentrations. DDT contains the sulfone moiety seen in DDS.

DDS-specific TCC cross react with 3DDS and low concentration of DDT dapsone analogues.

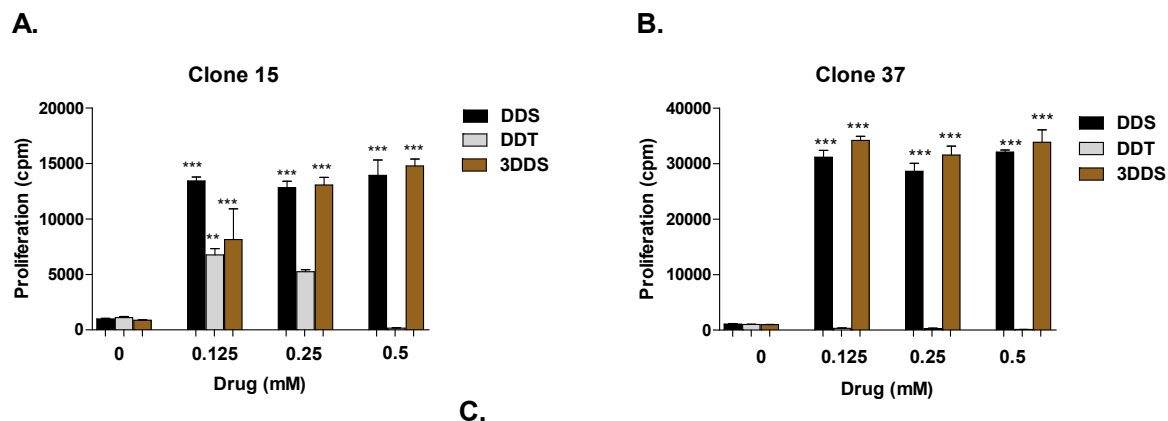


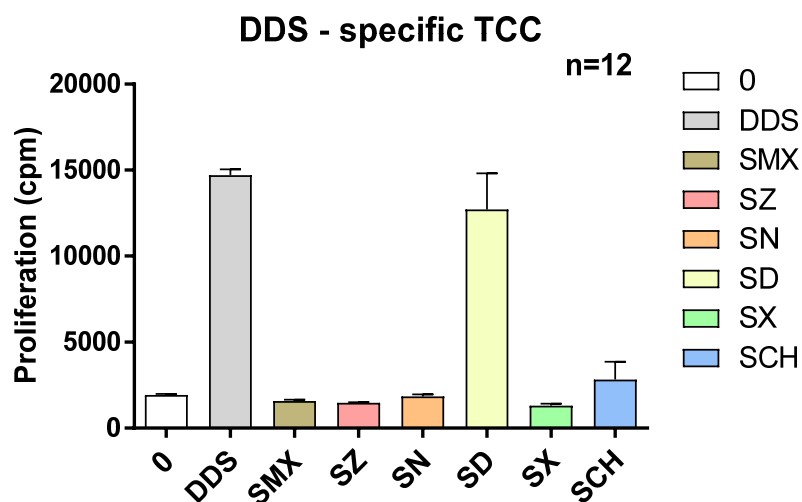
Figure 4.8: A, B) Cross reactivity of DDS-specific TCCs with closely related analogues: DDS-specific TCC were co-incubated with irradiated autologous EBV-transformed B-cells in the presence of DDS and closely related analogues in a 96 well plate at 5% CO₂ for 48 h. C) DDS analogues chemical structures.

4.3.8 Cross reactivity of DDS-specific TCC with sulfonamide antimicrobials.

12 clones generated from PBMC isolated from DDS-hypersensitive patients were used for the analysis. A high proliferative response was observed with DDS and sulfadiazine (SD) (Figure 4.9). All other compounds did not activate the clones.

DDS-specific TCC do not cross react with closely related sulphonamides except sulfadiazine(SD).

A.



B.

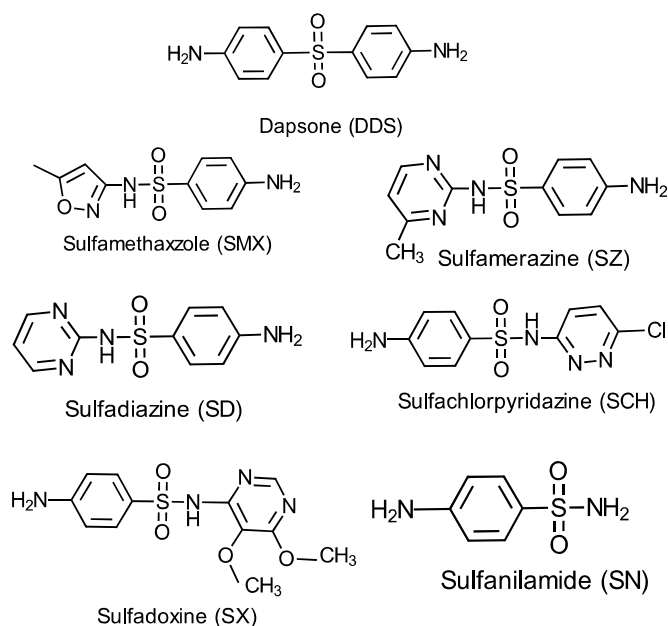


Figure 4.9: A) Cross reactivity of DDS-specific TCCs with closely related sulphonamides: DDS-specific TCC were co-incubated with irradiated autologous EBV-transformed B-cells in the presence of DDS or different sulphonamides (SMX, SZ,SD, SN, SX,SCH- 0.5 mM) in 96 wells plate at 5% CO₂ for 48 h. (B)sulfonamide structures and abbreviated names . Cross reactivity was assessed using thymidine proliferation assay.

4.3.9 Cross reactivity of TCC with DDS metabolites.

12 clones generated from PBMC isolated from DDS-hypersensitive patients were used for the analysis. A high proliferative response was observed with DDS hydroxylamine and DDS-NO for DDS-specific-TCCs as shown in (Figure 4.10A). The DDS-NO-specific TCC cross reacted with DDS hydroxylamine but not with parent drug (Figure 4.10).

DDS-specific TCC cross react with dapsone hydroxylamine and nitroso-dapsone.

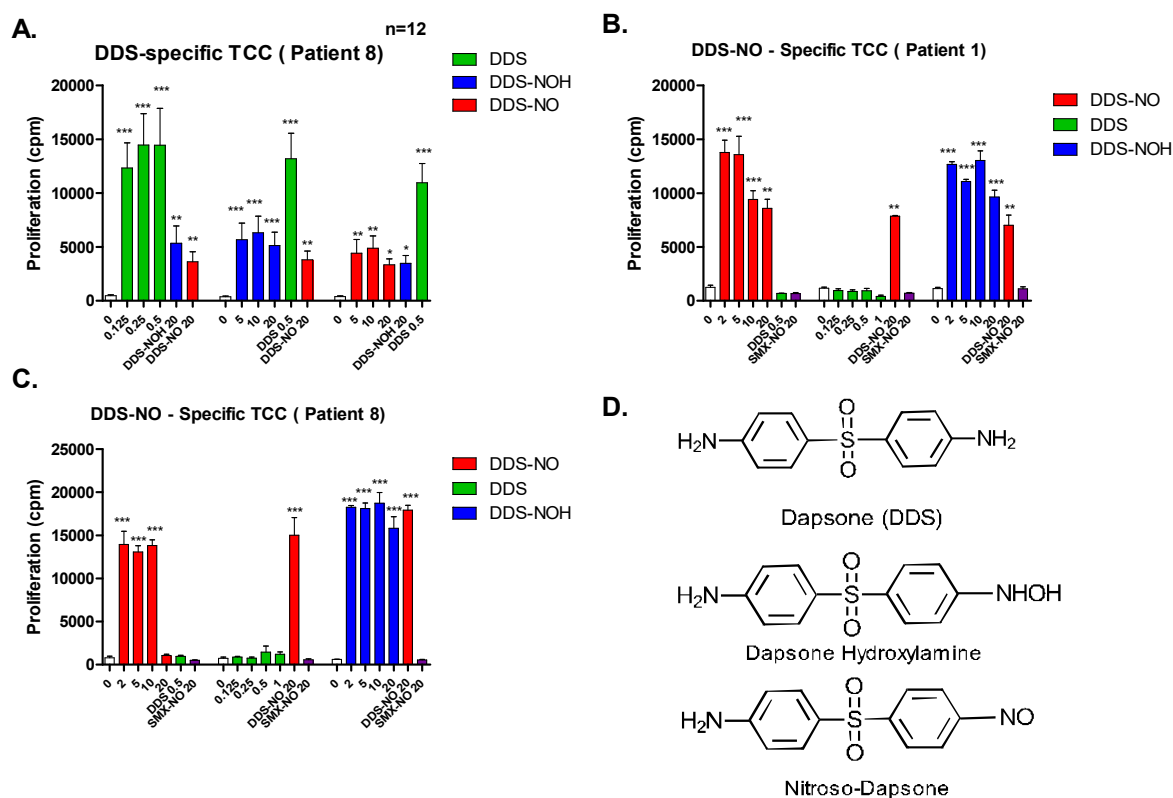


Figure 4.10: A) Cross reactivity of DDS-specific TCCs with closely related metabolites: DDS-specific TCC was co-incubated with irradiated autologous EBV-transformed B-cells in the presence of DDS or different metabolites (DDS-NO and DDS-NOH) 20 μ M) in 96 wells plate at 5% CO₂ for 48 h. B, C) Cross reactivity of DDS-NO-specific TCCs with closely related metabolite and parent drug (DDS, DDS-NOH). (D) Metabolites abbreviation and structure. Cross reactivity was then assessed using thymidine proliferation assay.

4.3.10 Glutathione inhibits activation of DDS-NO-specific, but not DDS-specific TCC.

Glutathione acts as an antioxidant. It exists in high levels in most cells and tissues and plays a significant role in metabolism as well as for and removal of xenobiotic substances (Perricone et al. 2009). Our results shown glutathione reduces in the proliferative responses of DDS-NO-specific TCC, but not the activation of DDS-specific TCC (Figure 4.11).

Glutathione terminates the activation of DDS-NO-specific TCC.

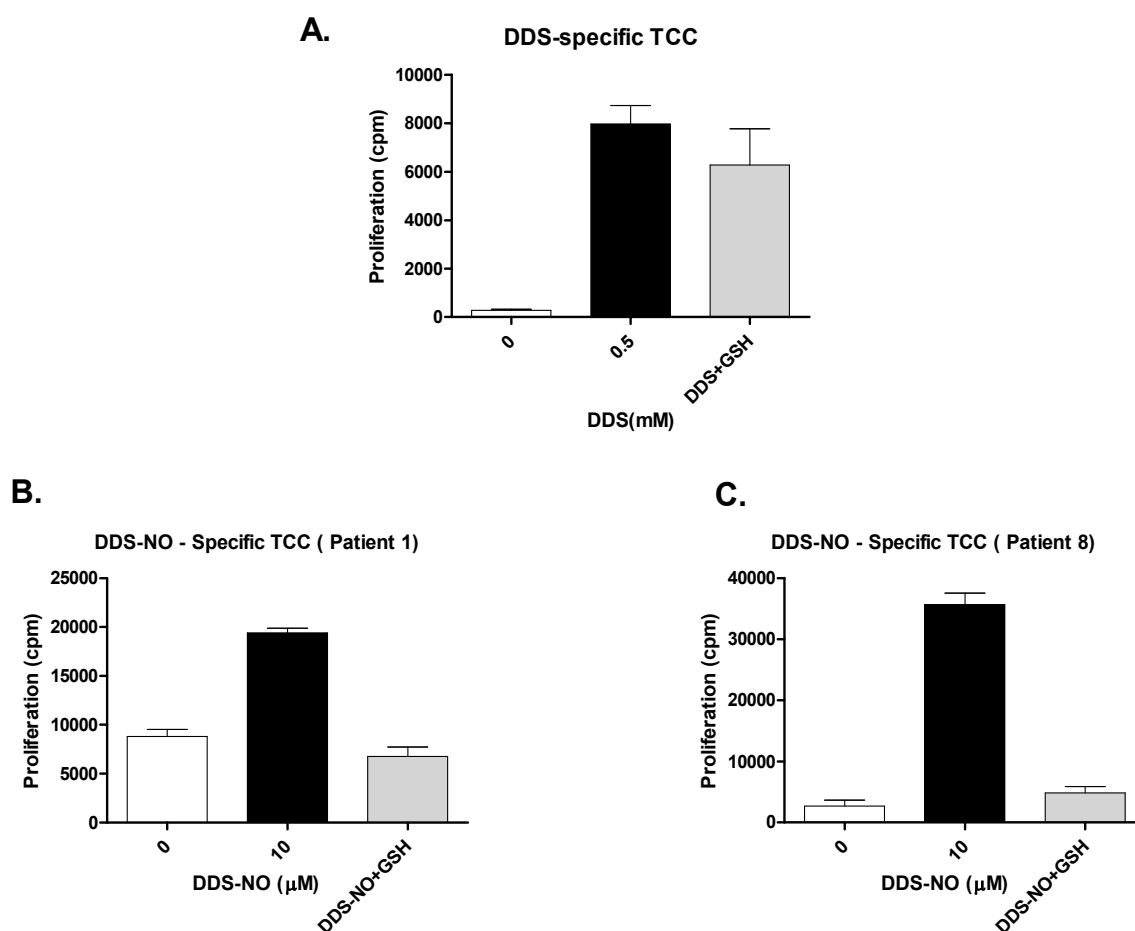


Figure 4.11: Activation of TCC with DDS and DDS-NO in the presence and absence of glutathione A) DDS-specific and B, C) DDS-NO TCCs were co-cultured with irradiated autologous EBV-transformed B-cells and DDS or DDS-NO in a 96 well plate at 5% CO₂ for 48 h. Proliferation was determined using radioactive thymidine incorporation during the final 16 hours of incubation and T cell proliferation evaluated using scintillation counting.

4.3.11 Molecular mechanism for activation of DDS and DDS-NO-specific TCC.

Anti-human MHC I and MHC II blocking antibodies were used to determine MHC-restricted T-lymphocyte activation in proliferation and ELISpot assays. Proliferation of both DDS and DDS-NO specific TCC was significantly decreased in the presence of an MHC II blocking antibody (Figure 4.12A, B&C). Furthermore, EBV-transformed B-cells pulsed with DDS (0.5 mM) for 16 hours, failed to induce proliferation and interferon-gamma secretion from DDS-specific TCC (Figure 4.12A). In contrast two DDS-NO-specific TCC were activated with APC pulsed with the nitroso metabolites (Figure 4.12B&C). Finally, APC fixed with glutaraldehyde before incubation with DDS (0.5 mM) induced the activation DDS-specific TCC proliferation and interferon-gamma secretion, whereas the response of two DDS-NO-specific reduced (Figure 4.12A, B&C).

Molecular mechanism of DDS and DDS-NO-specific TCC activation.

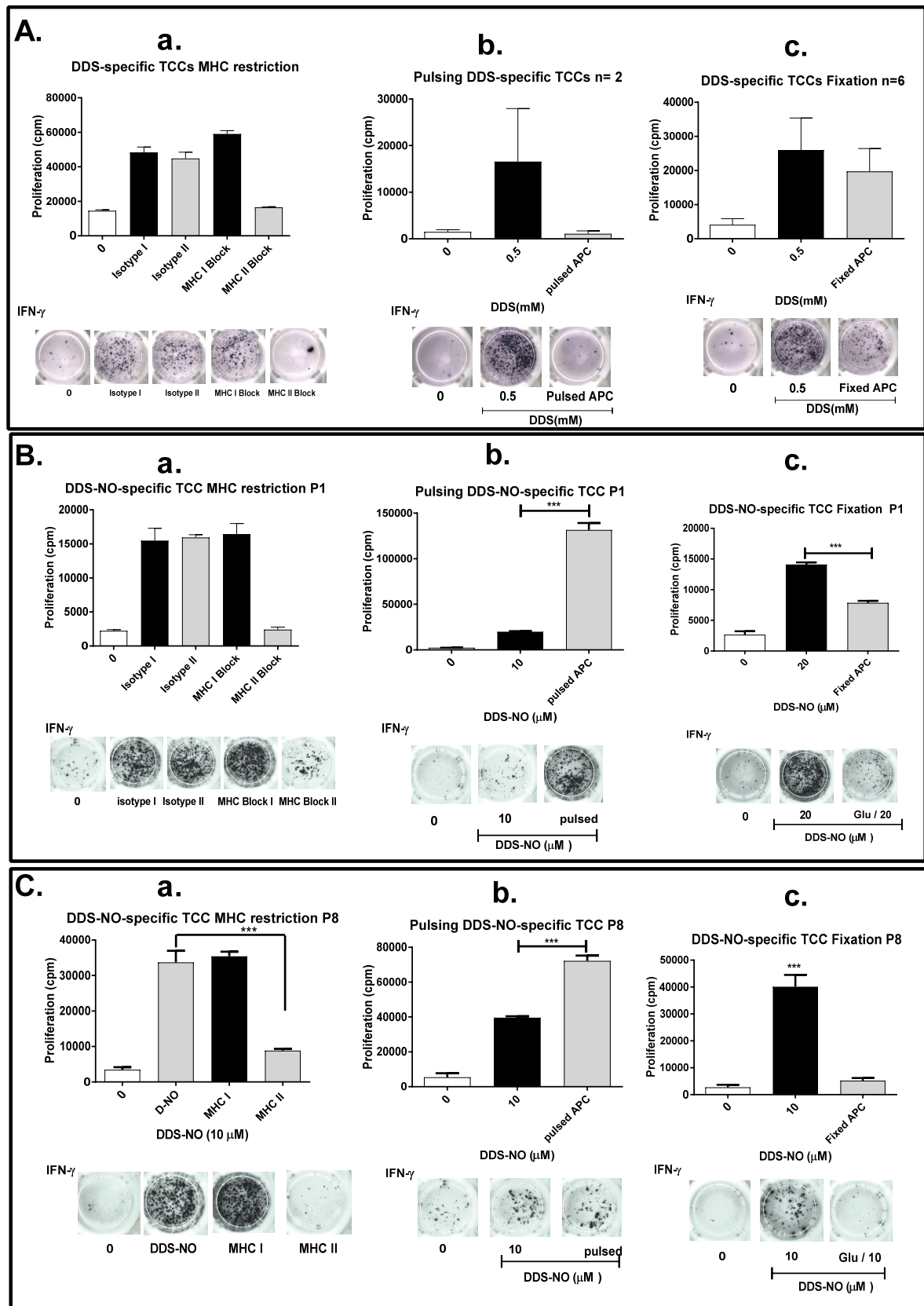


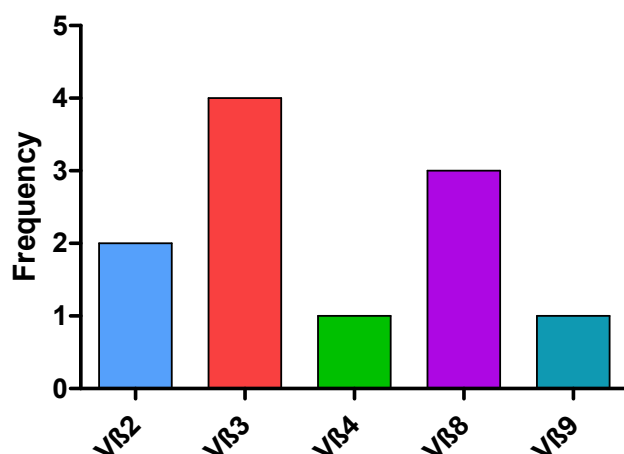
Figure 4.12: A) DDS-specific TCCs generated from one DHS patient. Proliferation was measured using [^3H] thymidine. ELISpot plates were pre-coated with human IFN- γ coating antibodies according to manufacturer's instruction and incubated overnight at 4°C to measure cytokines release from the TCC. TCCs were co-incubated with irradiated autologous EBV-transformed B-cells and DDS (0.125 -1 mM) using culture medium as negative control in the presence either MHC I or MHC II blocking antibodies. The plates were incubated at 37°C, 5% CO $_2$ for 48 h and plates developed for image visualisation and spot counts. B. EBV-transformed B-cells were pulsed with DDS for 24h and cells washed extensively to remove free drug. Drug-pulsed EBVs were then co-cultured with DDS-specific TCC, and proliferation and IFN- γ secretion determined as described above. C. EBVs were first treated with glutaraldehyde to abolish antigen processing then co-cultured with DDS-specific TCC and DDS followed by assessment proliferation and IFN- γ ELISpot assay. B,C) DDS-NO-specific TCCs generated from two patients, MHC restriction, pulsing and Fixation assays were performed as describe above.

4.3.12 T-cell V β receptor analysis.

Examination of T-cell receptor expression on 11 DDS-specific TCC generated from patient 8 revealed a distribution of TCR expression across five V β subclasses. TCCs expressed five different V β repertoires (V β 2, V β 3, V β 4, V β 8 and V β 9; Figure 4.13A). The percentage of V β T-cell receptor usage observed V β 2 (18%), V β 3 (37%), V β 4 (9%), V β 8 (27%) and V β 9 (9%) is shown in Figure 4.13B.

T cell V β receptor on DDS-specific TCC.

A. TCR frequency for DDS-specific TCC from P8



B. Percentage V β usage for DDS-specific TCC from P8

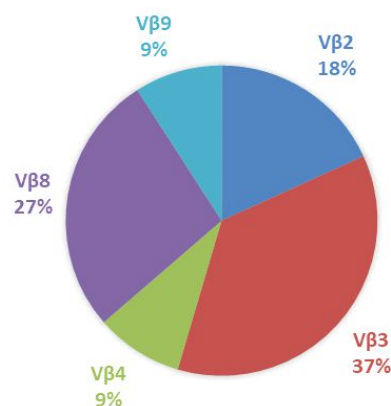
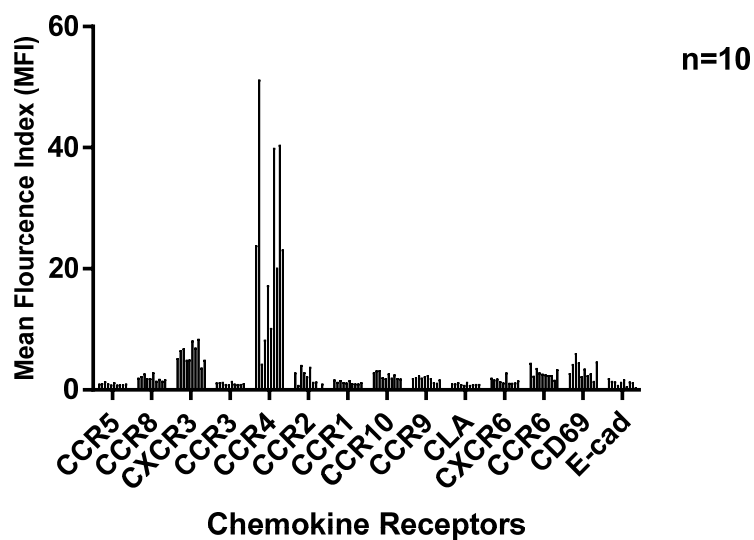


Figure 4.13- TCR V β analysis. T-cell suspensions (100 μL) were incubated with various TCR V β antibodies and TCR V β usage determined using flow cytometry and data analyzed. (A) Frequency graphical representation of TCR V β usage of DDS-specific TCC generated from 1 DDS-hypersensitive patient (P8). (B) Percentage TCR V β expression of 11 DDS-specific TCC.

4.3.13 Chemokines receptor analysis.

Cell phenotyping was performed by flow cytometry on a BD FACSCanto II using CCR1, CCR2, CCR3, CCR4, CCR5, CCR8, CCR9, CCR10, CXCR3, CXCR6, and CLA antibodies (BD Biosciences). Ten DDS-specific clones with a strong growth pattern were selected to explore which clones expressed specific chemokines receptors and hence have the ability to migrate towards tissues. All clones expressed high level of CXCR3 and CCR4 (Figure 4.14A& B). Other receptors were expressed at lower level. Clones migrated in response to CCL17 but not CCL27 (Figure 4.14 C).

A.**Chemokine Receptor Analysis of DDS-specific TCC****B. Table : chemokine receptor analysis for 10 DD-specific TCC.**

	1	2	3	4	5	6	7	8	9	10
CCR5	-	-	-	-	-	-	-	-	-	-
CCR8	+	+	+	+	+	+	-	-	-	-
CXCR3	+	+	+	+	+	+	+	+	+	+
CCR3	-	-	-	-	-	-	-	-	-	-
CCR4	+	+	+	+	+	+	+	+	+	+
CCR2	+	-	+	+	+	+	-	-	-	-
CCR1	-	-	-	-	-	-	-	-	-	-
CCR10	+	+	+	+	+	+	+	+	+	-
CCR9	+	+	+	+	+	+	+	-	-	-
CLA	-	-	-	-	-	-	-	-	-	-
CXCR6	+	-	-	-	-	+	-	-	-	-
CCR6	+	+	+	+	+	+	+	+	-	+
CD69	+	+	+	+	+	+	+	+	-	+
E- cad	-	-	-	-	-	-	-	-	-	-

C. TCCs migrate across a transwell membrane towards chemokines

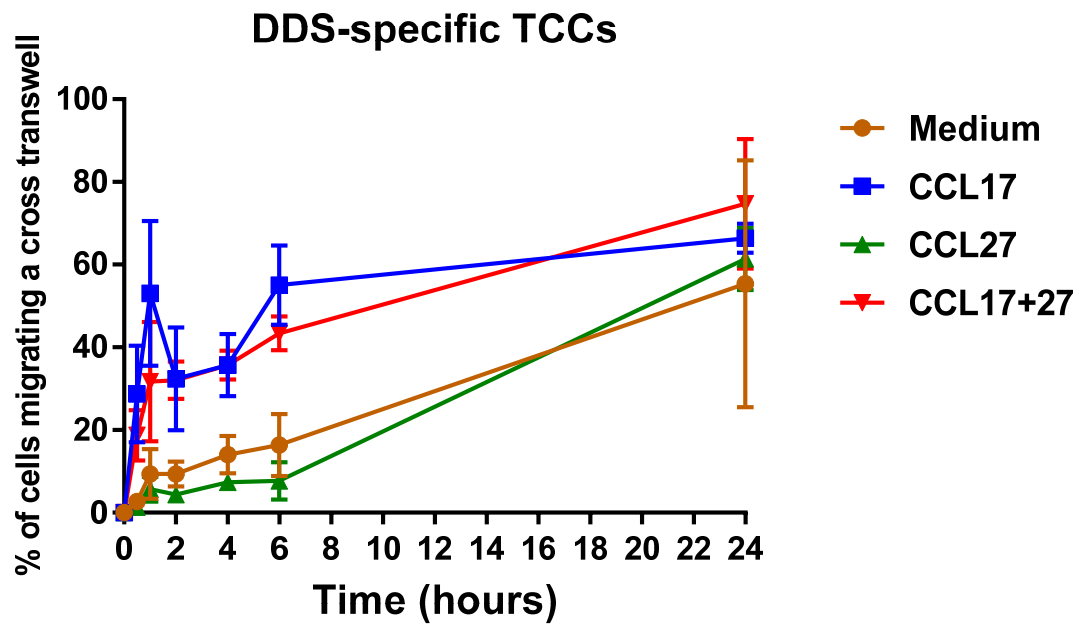


Figure 4.14 Chemokines receptor analysis. T-cell suspensions (100 μ L) were incubated with various TCR V β antibodies and TCR V β usage determined using flow cytometry and data analyzed by cyflogic. (A) Frequency graphical representation of TCR V β usage of DDS-specific TCC generated from DDS-hypersensitive patients. (B) Percentage TCR V β expression of 11 DDS-specific TCC. (C) TCC migration towards CCL17/or CCL27.

4.4 Discussion

DDS has been used for the treatment of leprosy and other diseases for a number of years. The use of DDS may lead to a hypersensitivity reaction known as drug hypersensitivity syndrome. There are number of factors involved in DDS hypersensitivity syndrome and these factors are genetic and non-genetic. Several studies have concentrated on the role of the HLA allele in drug-induced hypersensitivity reactions (Chung et al. 2008; Mallal et al. 2008; Daly et al. 2009; Illing et al. 2012; Yun et al. 2012). The most important HLA gene involved in DDS hypersensitivity is HLA-B*13:01 (Zhang et al. 2013). Further risk factors that may relate to hypersensitivity include: the chemical structure of the drug (Guglielmi et al. 2006), viral infections such as HIV and herpes viruses (Coopman et al. 1993; Shiohara et al. 2006), gender (Schmid et al. 2006; Thong and Tan 2011), genetic predisposition other than HLA (Kim et al. 2010) and T-cell receptor repertoire (Ko et al. 2011).

To investigate the molecular mechanism of dapsone hypersensitivity, we synthesized DDS-NO by the chemical oxidation of DDS hydroxylamine as described in chapter 2. 6 DHS patients were recruited from China and several experiments such as LTT and T-cell cloning were performed to assess the nature of the drug that activates T-cell, cross reactivity, MHC restriction and mechanism of drug-specific T-cell activation.

Lymphocyte from the hypersensitive patients were activated with both DDS and DDS-NO. This may be due to cross reactivity or the selective activation of individual clones by DDS and DDS-NO. To investigate this further, TCC were generated from dapsone hypersensitive patients.

Using the serial dilution method to generate drug-specific TCC from DHS patients, we generated twelve DDS-specific clones and one DDS-NO-specific TCC from patient 8. One DDS-NO responsive clone was generated from patient 1, with SI ranging from 2 to 120 as

shown in Figure 4.3. 50% of the twelve DDS-specific clones tested showed cross-reactivity with DDS-NO (Figure 4.4) but not with nitroso sulfamethoxazole. In contrast, both DDS-NO-specific clones showed no cross reactivity with parent drug or nitroso sulfamethoxazole. Furthermore, both DDS-specific clones and DDS-NO specific TCCs expressed the CD4⁺ cell surface protein (Figure 4.5). With respect to cytokine secretion profile, both DDS and DDS-NO-specific TCC proliferated and secreted IFN- γ , IL-5, IL-13, and GB, IL-22 following drug stimulation (Figure 4.6). Somewhat surprisingly, only few DDS-NO-specific clones were generated. Furthermore, CD8⁺ clones activated with DDS or DDS-NO were not detected. In subsequent experiments since the completion of this thesis CD8⁺ T-cells have been separated prior to serial dilution after a 2 week culture period with DDS and DDS-NO. Researchers have successfully identified large number of CD8⁺ clones activated with both DDS and DDS-NO and are actively working to investigate whether the drug antigens bind selectively to HLA-B* 13:01 to activate these T-cells.

In order to evaluate the role of APC in the stimulation of DDS and DDS-NO-specific TCC, we stimulated the TCC in presence or absence of irradiated autologous EBVs which act as APC. APC were required for the activation of DDS and DDS-NO-specific TCC (Figure 4.7).

To determine, whether DDS-specific TCCs cross reacted with closely related sulfonamides, we evaluated the reactivity of DDS-specific clones with other sulfonamide compounds which included sulfamethoxazole, sulfamerazine, sulfadiazine, sulfanilamide, sulfadoxin and sulfachloropyridazine. In a similar experiment, closely DDS analogues namely 4,4 diaminodiphenyl sulfide (DDT) and 3,3 -diaminodiphenyl sulfone (3DDS) were also tested. All compounds were used at a optimised dose of 0.5mM. 3DDS which has structure similar to DDS was cross-reactive with DDS-specific TCC (Figure 4.8). In addition the DDS-specific TCC cross-reacted with both DDS metabolites namely DDS-NO and DDS hydroxylamine (Figure 4.10). None of the TCC tested showed cross-reactivity with sulfonamides with exception of

the sulfadiazine (Figure 4.9). These data indicate that the sulfone group and at least one aromatic aniline moiety is needed to activate the TCC. Furthermore, the interaction is highly specific as subtle side chain modification beheads the T-cell response.

To characterise the HLA molecules involved in the presentation of drug antigen to DDS- and DDS-NO-specific TCC an MHC restriction assay was performed. This involves culturing antigen presenting cells, TCCs and drug in the presence of MHC class I and II blocking antibodies or isotype control and measuring the antigen-specific responses. The activation of DDS and DDS-NO-specific clones, whither were all CD4+, was MHC class II restricted (Figure 4.12).

To investigate further the mechanism of activation of DDS-specific TCC we performed an APC pulsing assay. Pulsed APC failed to stimulate DDS-specific TCC (Figure 4.12). In contrast, DDS-NO-specific pulsed APC succeeded to stimulate DDS-NO TTCs (Figure 4.12). These data suggested that DDS-NO binds covalently to APCs to activate T-cells, whereas the APC binding between DDS and MHC is labile and therefore reversible.

Antigen presenting cell fixation abolishes the ability of APCs to take up and/or to process protein. In this study APC were fixed with glutaraldehyde and used to stimulate the DDS and DDS-NO-specific clones with addition of soluble DDS or DDS-NO. Fixation of APC did not abolish the activation of DDS-specific TCC but the response of DDS-NO TCC was inhibited (Figure 4.12). Thus activation of DDS-NO-specific clones is dependent on protein processing with APC. TCRs are believed to play a significant role in the of T-cell reactivity to drug such as sulfamethoxazole and other structurally related compounds (Depta et al. 2004; Schmid et al. 2006). Therefore, we explored the distribution of TCR repertoire on 11 DDS-responsive TCC generated from 1 patient. DDS-specific TCC expressed 5 different TCRV_β receptors (V_β2, V_β3, V_β4, V_β8 and V_β9; Figure 4.13A). The majority of clones analysed expressed V_β3 (37%). The

V β 2 has been associated in a number of immune-related diseases and cutaneous allergic reactions (Reantragoon et al. 2012; Watkins and Pichler 2013). According to silico docking studies the CDR2 and CDR3 regions of sulfamethoxazole-responsive clones needed to express TCRV β 2 for an interaction with sulfamethoxazole (Watkins and Pichler 2013). Chemokines play critical role in the regulation the steady-state and inflammatory migration of cutaneous dendritic cells (DCs). We analysed chemokine receptors which including; CCR1, CCR2, CCR3, CCR4, CCR5, CCR8, CCR9, CCR10, CXCR3, CXCR6, and CLA on 10 DDS-responsive clones. Our data revealed most of clones expressed CCR4 (Figure 4.14), a skin homing receptor. Moreover, clones migrated toward the CCR4 ligand CCL17.

In summary, data presented in this chapter shows that CD4⁺ T-cells isolated from DDS hypersensitive patients are activated with both DDS and the activate metabolites DDS-NO via different mechanisms. On-going experiments are attempting to investigate whether CD8⁺ clones are also activated by both forms of the drug antigen.

Chapter 5: Definition of the antigenicity and immunogenicity of dapsone, sulfamethaxazole of their metabolites in a mouse model.

Contents

5.1 Introduction	144
5.1.1 Previous mouse immunogenicity studies	145
5.2 Aim of this study	147
5.3 Material and Methods	147
5.3.1 Animals	147
5.3.2 Drug administration	148
5.3.3 <i>In vitro</i> modification of His-GSTP with nitroso metabolites	148
5.3.4 <i>Ex Vivo</i> Proliferation of Splenocytes to test Drugs	149
5.3.5 Mass spectrometric analysis of DDS-NO and SMX-NO binding to mouse serum albumin	149
5.4 Results	151
5.4.1 Splenocytes from immunized mice are activated with nitroso dapsone, but not the parent drug	151
5.4.2 SMX-NO and DDS-NO modification of cysteine in GSTP	153
5.4.3 SMX-NO and DDS-NO modification cysteine residue in mouse serum albumin	156
5.5 Discussion	159

5.1 Introduction

The use of dapsone (DDS) is associated with many adverse effects such as anemia, hemolysis, methemoglobinemia, and DDS hypersensitivity syndrome (Cucinell et al., 1972, Coleman et al., 1996). The toxic effects of DDS is caused by its metabolites (Vyas et al., 2006). It has been proposed that the drug induced-hypersensitivity syndrome is an immune mediated response which involves activation of T-lymphocytes against a derivative of the drug (Ben M'Rad et al., 2009). DDS hypersensitivity can cause irreversible organ damage and sometimes lead to death if not managed properly or recognized when it is too late (Singh et al. 2016). DDS hypersensitivity reactions can develop in several weeks after the initiation of the treatment, with reported incidences ranging from 0.5% to 3% (Zhang et al., 2013). The cutaneous manifestations include: exfoliative dermatitis, erythroderma, popular erythematosis, erythema multi-formed, pustular eruptions, toxic epidermal necrosis, and Stevens-Johnson syndrome (Roujeau 2013). The liver is the major organ involved in DDS metabolism. DDS is oxidized to the reactive metabolite, DDS-NO via a hydroxylamine intermediate. (Uetrecht 1992; Reilly et al. 2000; Uetrecht 1992; Vyas et al., 2006, Roychowdhury et al., 2007, Sharma and Uetrecht, 2014).

The diagnosis of DDS hypersensitivity is dependent on the clinical presentation, along with the history of exposure to DDS. The pathogenesis of DDS hypersensitivity could be linked to formation of DDS-NO-protein adducts provoking a T-cell response. The bioactivation of many drugs to their respective reactive metabolites has been put forward as a critical factor in immunological reactions. For instance, lamotrigine, phenytoin and carbamezipine are all associated with a high incidence of immune reactions and have all been reported to generate highly reactive arene oxide metabolites that form protein adducts, resulting (Madden et al., 1996; Maggs et al., 2000). In the previous chapters of this thesis DDS-NO was synthesized and

compared the immunogenicity of DDS and DDS-NO using PBMC from drug-naïve healthy donors and DDS hypersensitive patients. Both the parent drug and reactive nitroso metabolite activated T-cells in both experimental models. The current chapter focuses on the immunogenicity of DDS and DDS-NO in mice and compares the responses with those seen with nitroso sulfamethoxazole. The rodent system also allowed a comparison of the binding of DDS-NO and nitroso sulfamethoxazole to protein using mass spectrometry.

5.1.1 Previous mouse immunogenicity studies.

The use of animal models to investigate drug hypersensitivity reactions is poorly developed and requires further research. In a study carried out by Farrell et al.(2003) to determine the immunogenicity of sulfamethoxazole (SMX) in animals such as male rats, mice and rabbits, animal were immunized with test drugs and drug-specific splenocyte proliferation was evaluated. The metabolism of SMX involves CYP450 and myeloperoxidase-catalyzed reactions, which leads to the formation of a hydroxylamine that is not protein reactive (Farrell et.,al 2003). SMX hydroxylamine in the peripheral circulation undergoes auto-oxidation in circumstances of oxidative stress to generate protein-reactive nitroso metabolites similar to DDS-NO. SMX-NO is highly unstable in solution, and degrades to produce nitro SMX, dimerization product (azo and azoxy adducts) and is reduced to SMX hydroxylamine and SMX as summarized in the schematic representation below (Figure 5.1). Splenocytes from mice immunized against SMX-NO, but not the parent drug, are stimulated to proliferate with the nitroso metabolite, which indicates that SMX-NO is highly immunogenic in these species. The lack of a response to the parent drug highlights a clear difference between human and rodent models.

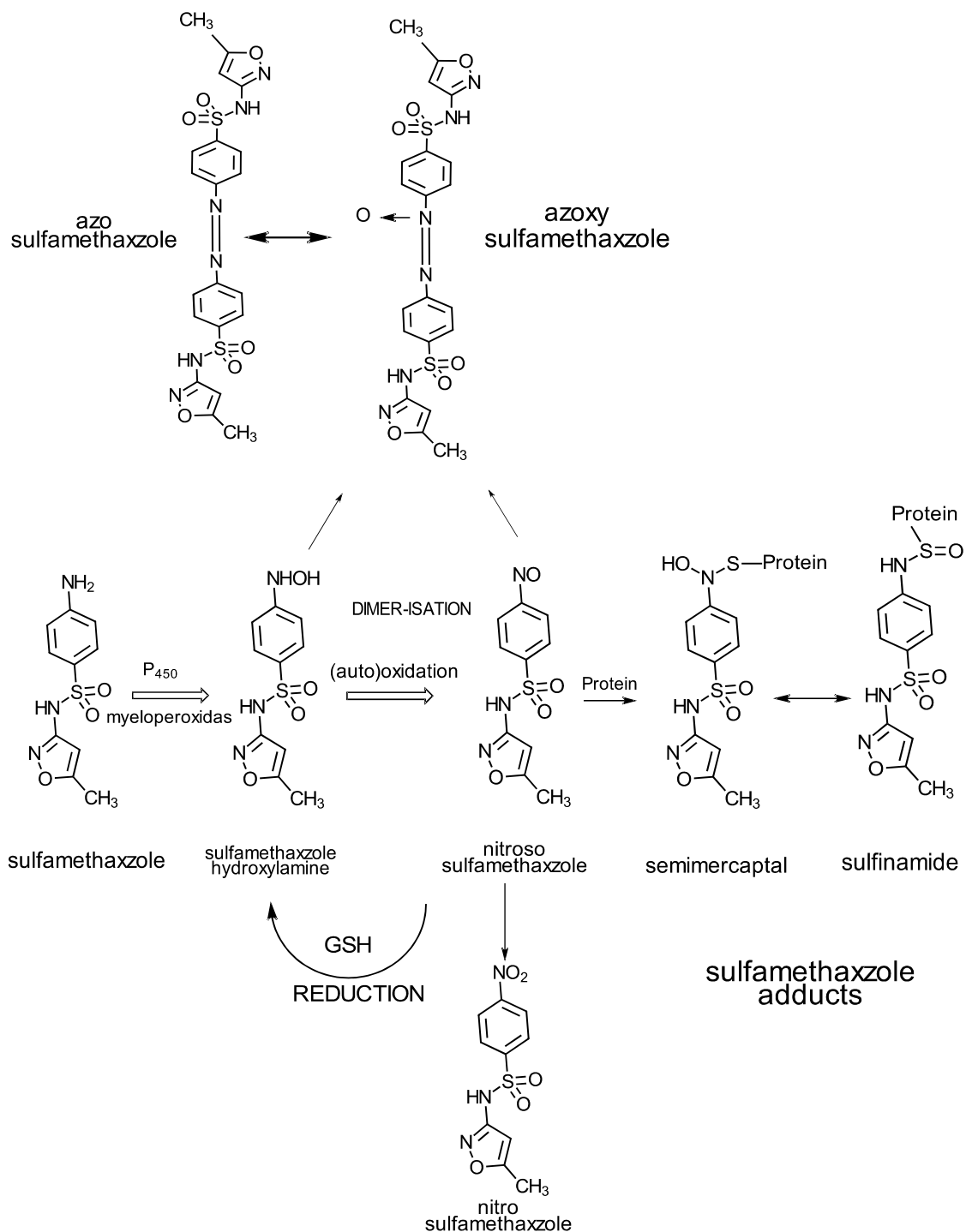


Figure 5.1 Scheme describing the SMX- and SMX-oxidative metabolites *in vivo* adapted from (Farrell et al. 2003).

According to Whritenour et al. (2014), animal models are useful tools for identifying drug candidates that activated immune cells (Whritenour et al. 2014). Other important animal models which have been developed to study hypersensitivity reactions are penicillamine-induced

autoimmunity in the Brown Norway Rat (Tournade et al, 1990), nevirapine-induced skin rash in the rat (Shenton et al., 2003), propylthiouracil-induced autoimmunity in the cat (Aucoin et al, 1985), sulfonamide-induced hypersensitivity in dogs (Trepanier,2004), amodiaquine-induced agranulocytosis/hepatotoxicity in rats (Clarke et al, 1990), isoniazid-induced hepatotoxicity in the rabbit (Sarich et al, 1995) and lipopolysaccharide-potentiated hepatotoxicity in rats (Luyendyk et al, 2006).

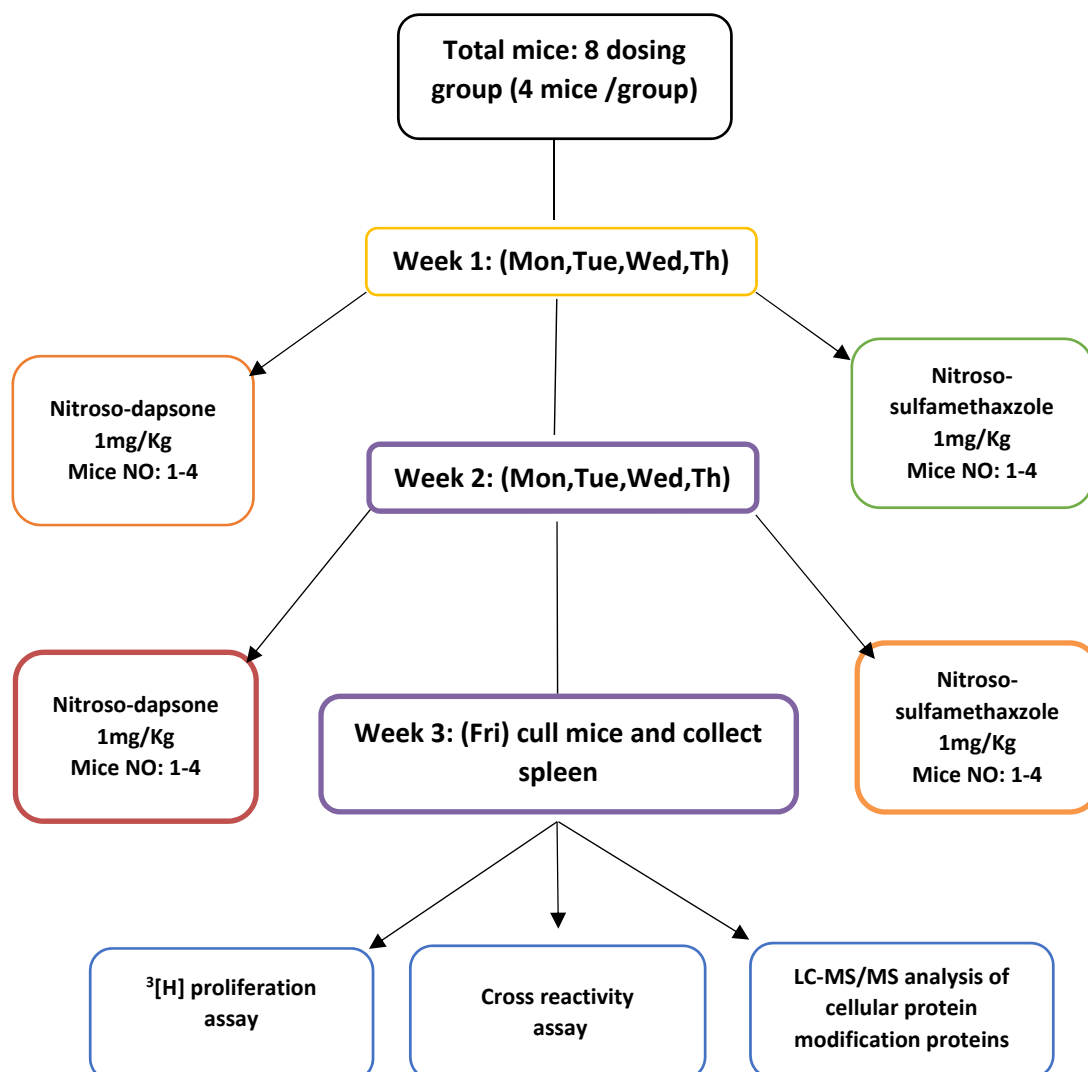
5.2 Aim of this study

- 1- Explore the immunogenicity/antigenicity of DDS, SMX and their nitroso metabolites on using mouse model.
- 2- Analysis of DDS-NO protein modification.

5.3 Material and Methods

5.3.1 Animals

Female C57BL/6 wild type mice, (8-12 week old), weighing 25-30g were purchased from Charles River (Wilmington, MA, USA). All mice used in this study were kept in the animal housing unit of Liverpool University. All mice were given access to food and water after arrival to the facility. All experiments were conducted under a project and personal license issued by the UK Home Office under the Animals (Scientific Procedures) Act 1986. Eight mice were divided into two groups with each group containing 4 mice. Every group was injected with the following: DDS-NO, or SMX-NO at (1mg/kg inj IP) once a day for four days in the first week. Dosing was repeated for second week. A week later mice were culled, spleen removed and splenocyte isolated for *in-vitro* experiments (Figure 5.2).

Schematic representation of dosing schedule and experiment design.**Figure 5.2:**Schematic representation of the mouse experiments**5.3.2 Drug administration**

All the drugs were dissolved in DMSO prior to intraperitoneal injection (1mg/kg) using a 27g needle.

5.3.3 *In vitro* modification of His-GSTP with nitroso metabolites

Expression and affinity purification of histidine-tagged human GSTP was carried out using protocols published previously (Jenkins et al.,2008). Briefly, the cDNA for histidine-tagged human GSTP (His-GSTP) was cloned into the vector pET-15b (Novagen) and expressed as

described previously. Transformed BL21 cells were grown on lysogeny broth (LB) medium containing ampicillin (50 µg/mL). A single colony was inoculated into 50 mL of LB medium and grown at 37°C until the absorbance at 600 nm reached approximately 0.5. An aliquot of this preculture was added to 1 L medium which was incubated on a shaker at room temperature. Protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 2 mM. After 18h, the cells were harvested by centrifugation at 4000 g for 5 min, and the cell pellets were resuspended in 10 mL phosphate buffer and sonicated. The sonicated cells were centrifuged at 13,000 rpm for 10 min and the supernatant was collected and stored at -80°C. Purified His-GSTP captured on nickel beads was incubated with a range of molar ratios of nitroso metabolites to protein (1:1-10:1) in phosphate buffer, pH 7.4 for 16 h. The beads were then washed with 5X100 µL phosphate buffer. The modified protein was subjected to on bead tryptic digestion followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

5.3.4 *Ex Vivo* Proliferation of Splenocytes to test Drugs.

Red cell-depleted splenocytes isolated by density centrifugation using lymphoprep were incubated (1.5×10^5) with DDS (0.1-0.5 mM), sulfamethoxazole (0.1-0.5 mM), DDS-NO (5-20 µM), SMX-NO (5-20 µM) and nitroso benzene (20 µM) at 37°C, 5% CO₂. After 3 days, proliferation was measured by the addition of [³H] thymidine for the final 16 h of culture.

5.3.5 Mass spectrometric analysis of DDS-NO and SMX-NO binding to mouse serum albumin.

To investigate the covalent binding of DDS-NO and SMX-NO to mouse serum albumin (MSA) (Figure 5.8), DDS-NO freshly dissolved in DMSO was incubated with MSA (0.1 mM, 50 µL) in potassium phosphate buffer (10 mM, pH 7.4) at 37 °C for 16 h. The molar ratios of drug to protein were 0.1:1, 1:1, and 10:1. Protein was purified by ultrafiltration (3K cutoff) to remove the free drug, and then reduced with 10 mM dithiothreitol (15 min) and alkylated with 55 mM

iodoacetamide (15 min) at room temperature. The protein was reconstituted in 100 μ L 50 mM ammonium hydrogencarbonate, and 165 μ g (1.25 nmol) of protein was digested with 1.6 μ g trypsin overnight at 37°C. The tryptic MSA peptides were analysed by a Q-TOF mass spectrometer as described previously (Jenkins et al. 2013). Briefly, samples were delivered into a Triple TOF 5600 mass spectrometer (AB Sciex) by automated in-line reversed phase liquid chromatography, using an Eksigent NanoUltra cHiPLC System (AB Sciex) mounted with a microfluidic trap and analytical column (15 cm \times 75 μ m) packed with ChromXP C18-CL 3 μ m. A NanoSpray III source was fitted with a 10 μ m inner diameter PicoTip emitter (New Objective). Samples were loaded in 0.1% formic acid onto the trap, which was then washed with 2% ACN/0.1% FA for 10 min at 2 μ L/min before switching in-line with the analytical column. A gradient of 2–50% (v/v) ACN/0.1% (v/v) FA over 90 min was applied to the column at a flow rate of 300 nL/min. Spectra were acquired automatically in positive ion mode using information-dependent acquisition, using mass ranges of 400–1600 amu in MS and 100–1400 amu in MS/MS. Up to 25 MS/MS spectra were acquired per cycle (approximately 10 Hz) using a threshold of 100 counts per s, with dynamic exclusion for 12 s and rolling collision energy. Sequence coverage was determined using ProteinPilot software, v4.0 and the most recent version of the SwissProt database. Modified peptides were identified by filtering for specific fragment ions in PeakView 1.2.0.3 (AB Sciex) and manual inspection of the spectra.

5. 4 Results

5.4.1 Splenocytes from immunized mice are activated with nitroso dapsons, but not the parent drug.

Previous studies have shown that splenocytes from mice immunized against SMX-NO are stimulated to proliferate *in vitro* in the presence of the nitroso metabolite. In contrast, splenocytes are not activated with the parent drug (Farrell et al., 2003). Herein, splenocytes from SMX-NO-sensitized mice were also shown to be activated with nitroso sulfamethoxazole and no cross-reactivity was observed with either DDS-NO, nitroso benzene or SMX (Figure 5.3B).

Mice were also administered DDS and DDS-NO following the protocol established with SMX-NO. Splenocytes from 2 DDS-NO immunized mice proliferated vigorously *ex vivo* in the presence of the nitroso metabolite (Figure 3A). Weaker DDS-NO-specific proliferative responses were detected with splenocytes from mice 3 and 4. Somewhat surprisingly, splenocytes from all 4 mice were also activated with SMX-NO. DDS and SMX did not activate the T-cells. Similarly splenocytes from mice immunized with DMSO alone were not activated with any of the compounds.

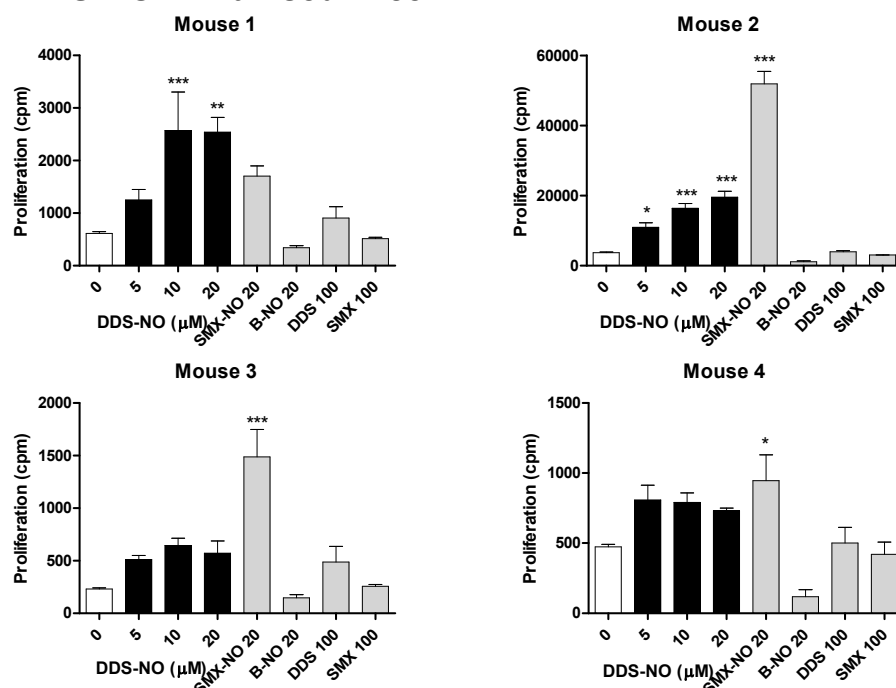
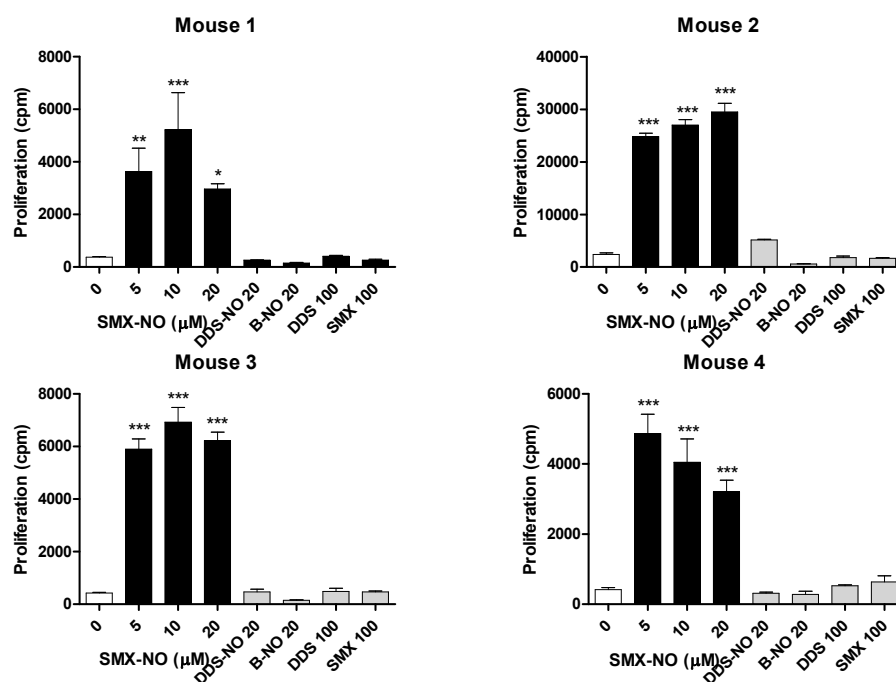
A . DDS-NO immunised mice**B. SMX-NO immunised mice**

Figure 5.3: Eight C57BL/6 mice were immunized with **A)** DDS-NO(1mg/kg) or **B)** SMX-NO (1mg/kg) once a day intraperitoneally for 4 days for 2 weeks. 7 days later splenocytes were isolated counted and cultured in 96 U-bottom well plates in the presence and absence of drugs. Cross reactivity with (DDS,SMX and B-NO) were performed. Culture media was used as a negative control. The plate was incubated at 37°C for 48 hours. Proliferation was assessed by adding [³H]thymidine (0.5μCi) during the final 16 hours of incubation and T-lymphocyte proliferation evaluated using scintillation counting.

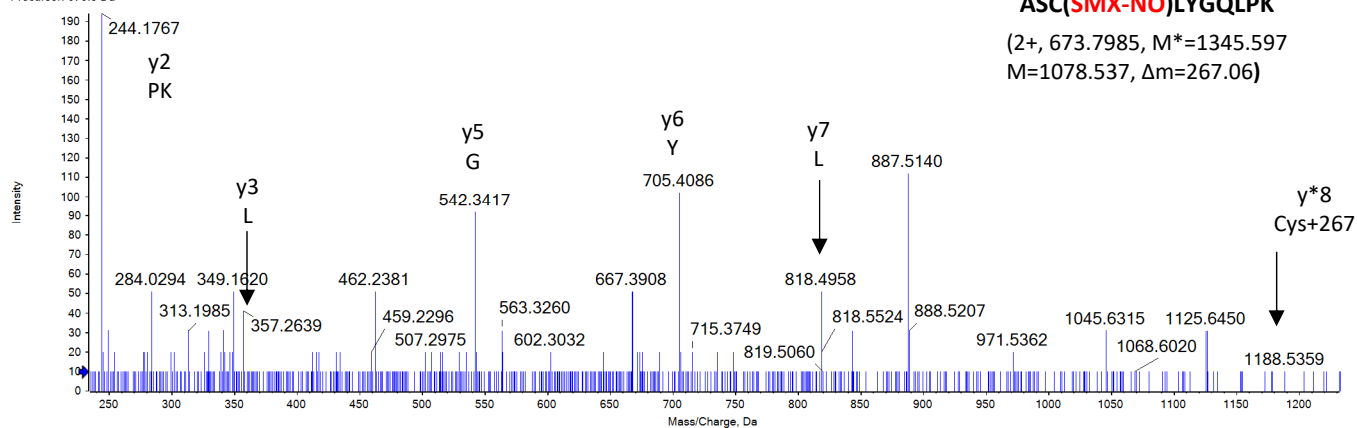
5.4.2 SMX-NO and DDS-NO modification of cysteine in GSTP.

To characterize the reactivity of nitroso metabolites with cysteine residues on proteins, human GSTP which contains several reactive cysteine residues was chosen as a model. His-GSTP captured on nickel beads was incubated with nitroso metabolites and the adducts were analyzed by LC-MS/MS. The modification of Cys47 by SMX-NO resulted in two types of adduct, corresponding to doubly charged ions at m/z 673.8 and 681.8, respectively. These adducts with a mass increase of 267 and 283 Da, were deduced to a sulfonamide and N-hydroxyl sulfonamide adducts, respectively (Figure 5.4A and 5.4B).

Similar to the modification observed with SMX-NO, modification of GSTP by DDS-NO resulted in a sulfonamide and N-hydroxyl sulfonamide adduct with a mass addition of 278 and 294, respectively (Figure 5 C-D). Interestingly, a third adduct with a mass addition of 262 was also detectable (Figure 5B). We deduced that this adduct could correspond to a sulfinamide, which is normally not stable and can be further oxidized to a sulfonamide. The modification of Cys47 by DDS-NO was confirmed by the MS/MS spectra in Figure 5 (B-D). In contrast to DDS-NO, no modification at Cys47 was observed when DDS was incubated with GSTP (Figure 5.5A).

A) Modification Cys47(GSTP) by SMX-NO

Spectrum from 161028 AAT SMX GSTpi 1 1.wiff (sample 1) - 161028 AAT SMX GSTpi 1 1, Experiment 17, +TOF MS² (100 - 1400) from 43.233 min
Precursor: 673.8 Da



B) Modification Cys47(GSTP) by SMX-NO

Spectrum from 161028 AAT SMX GSTpi 5 1.wiff (sample 1) - 161028 AAT SMX GSTpi 5 1, Experiment 11, +TOF MS² (100 - 1400) from 46.398 min
Precursor: 681.8 Da

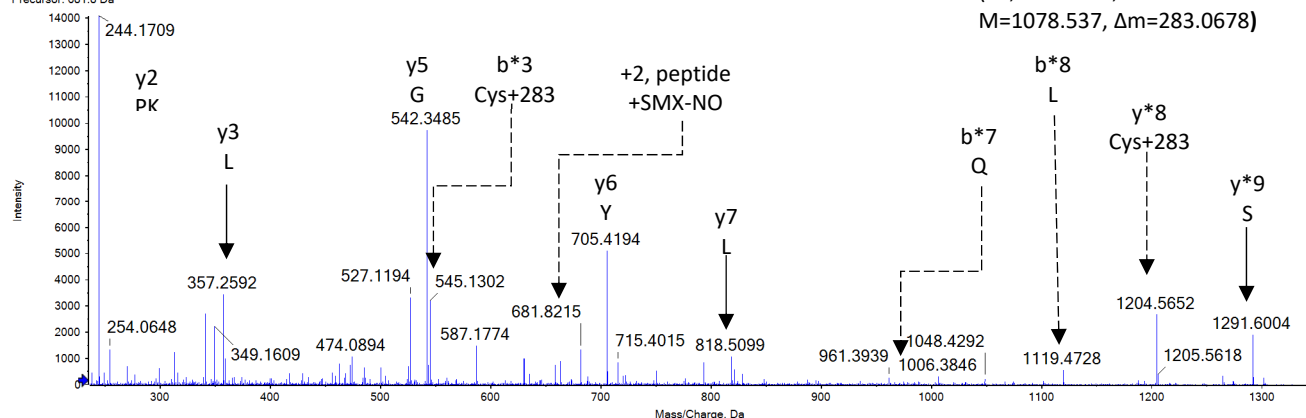


Figure 5.4: MS/MS spectra showing adduction of reactive metabolite (SMX-NO) to GSTP . SMX-NO covalently bound to GSTP peptide ASCLYGQLPK at Cys47 and resulted in a sulfonamide (A) and N-hydroxyl sulfonamide (B) adduct with a mass addition of 267 Da and 283 Da, respectively.

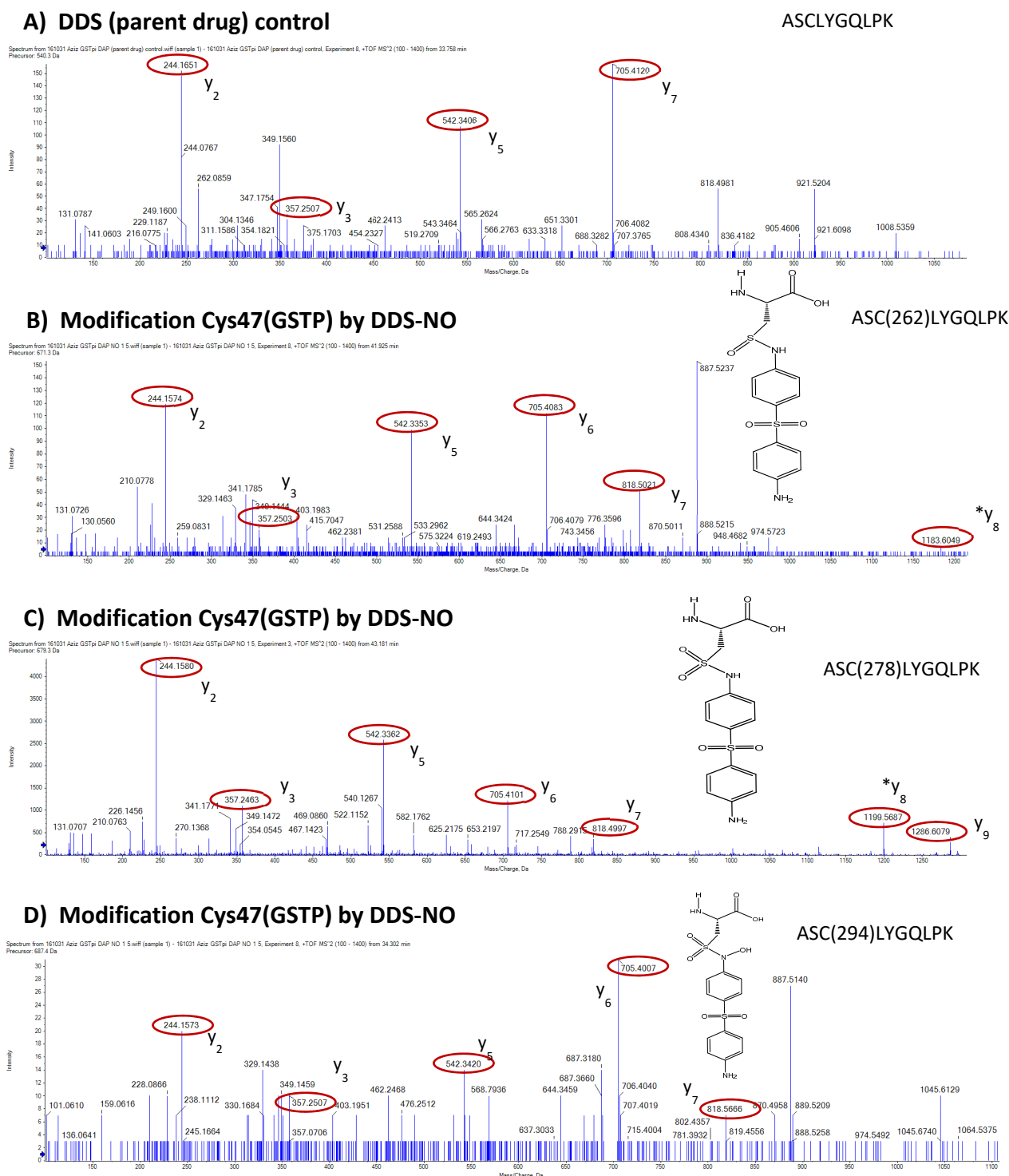


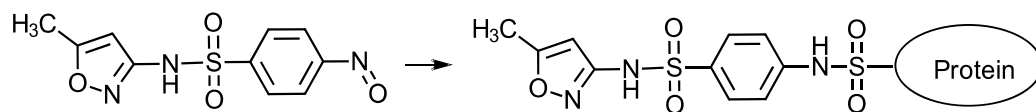
Figure 5.5: LC-MS/MS analysis of DDS or DDS-NO modified GSTP peptides identified in vitro. MS/MS spectra showing no DDS adducts formed on peptide ASCLYGQLPK (A), and the formation of sulfinamide, sulfonamide, and N-hydroxyl sulfonamide adducts with Cys47 on GSTP with a mass addition of 262 Da (B), 278 Da (C), and 294 Da (D), respectively. Peaks indicated correspond to m/z of y ions derived from peptides ASCLYGQLPK.

5.4.3 SMX-NO and DDS-NO modification cysteine residue in mouse serum albumin.

SMX-NO has previously been shown to undergo multiple adduction reactions with cysteinyl residues of proteins such as human serum albumin and GSTp. GSTp was modified at Cys47 forming sulfinamide, N-hydroxysulfinamide, and N-hydroxysulfonamide adducts. Human serum albumin was modified with nitroso sulfamethoxazole at Cys34 and in contrast to glutathione S-transferase pi, only the [2O] adduct was detected (Figure 5.6)

Herein, DDS-NO and SMX-NO were incubated with mouse albumin for 16h and adduct formation was measured by mass spectrometry. Similar to the [2O] adduct observed with SMX-NO on MSA (Figure 5.6), stable DDS-NO cysteine adducts on MSA were detected (Figure 7). A representative MS/MS spectrum for a doubly charged ion at m/z 615.72 corresponds to the tryptic peptide $^{34}\text{CSYDEHAK}^{41}$ with an additional mass of 278amu, indicating that a [2O] adduct was formed. The peptide sequence was confirmed by the presence of a series of y and b ions. The modification site was confirmed by b2* (m/z 469.1), b3* (m/z 632.2), and b6* (m/z 1013.3), and b7* (m/z 1084.3), all with adduction of 278 amu, giving evidence of modification at Cys34 (Figure 5.7).

A) Modification Cys34(MSA) by SMX-NO



Spectrum from Aziz SNO MSA 4h 100217.wiff (sample 1) - Aziz SNO MSA 4h 100217, Experiment 9, +TOF MS² (100 - 1400) from 21.181 min
Precursor: 618.2 Da, CE: 10.0

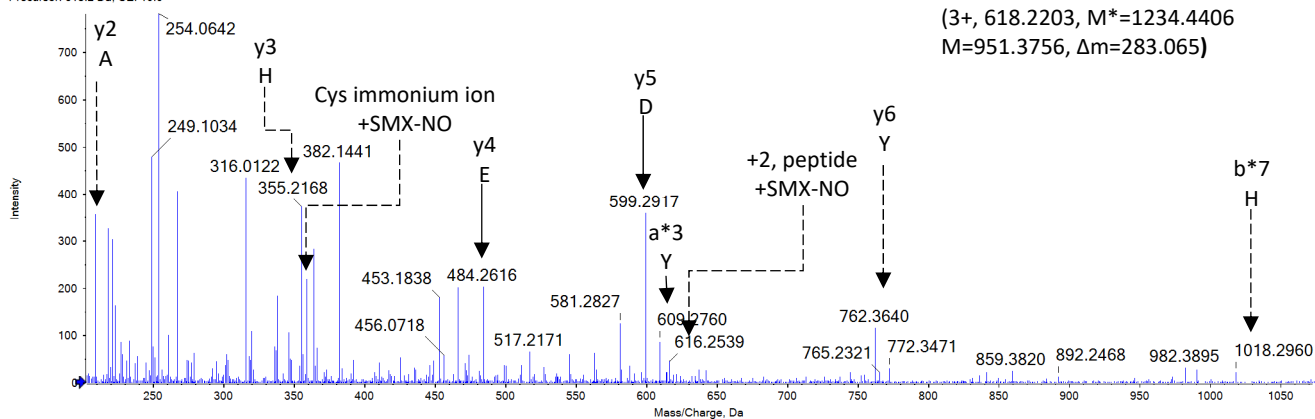


Figure 5.6: Mass spectrometric detection of the SMX-NO adduct formed with MSA, Illustrative MS/MS spectra of peptide 34CSYDEHAK41 modified on cystine34.

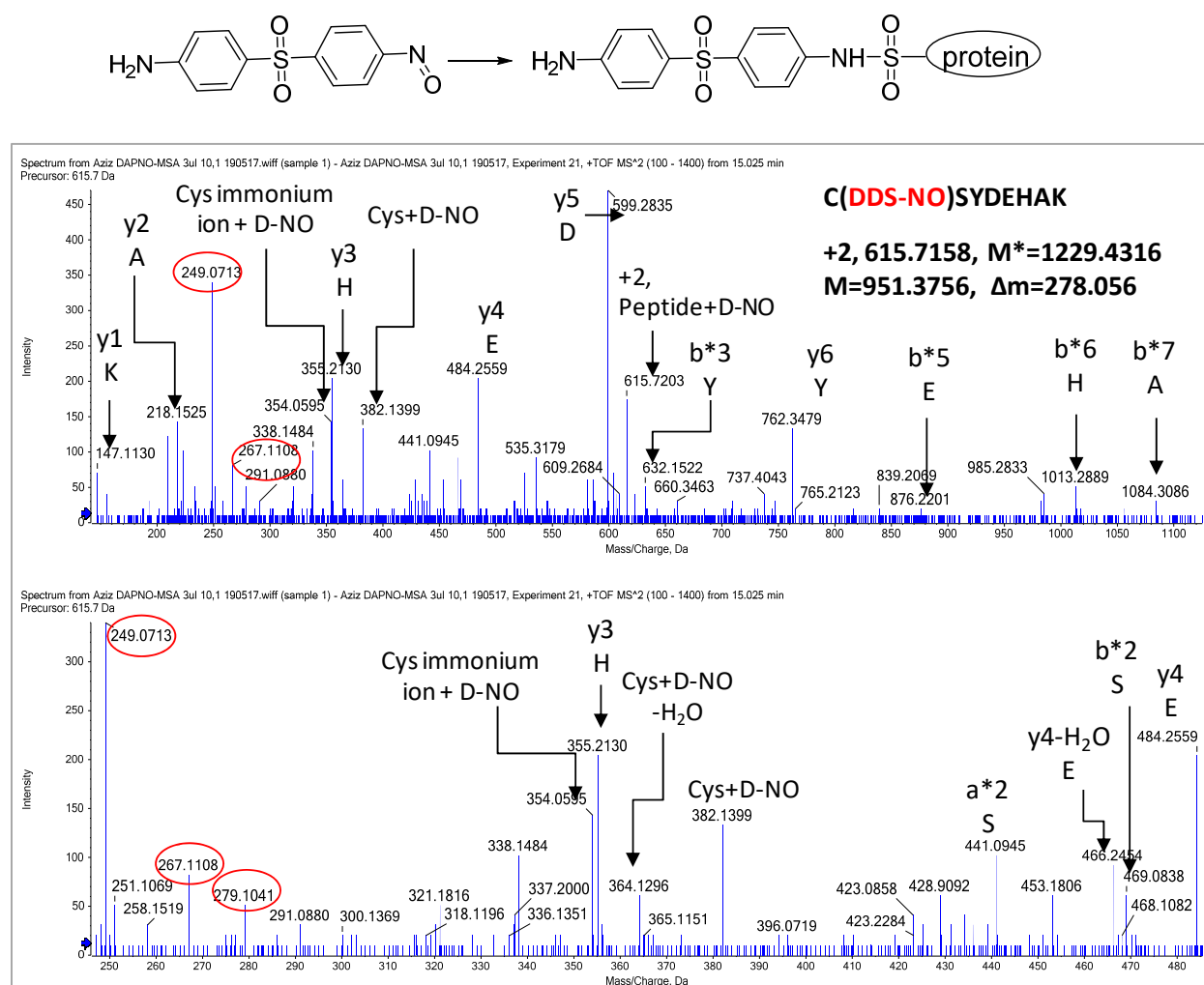


Figure 5.7: Mass spectrometric detection of DDS-NO adduct formed with MSA, Illustrative MS/MS spectra of peptide 34CSYDEHAK41 modified on cystine34.

MKWVTFLLLLFVSGSAFSRGRVFRREAHKSEIAHRYNDLGEQHFGLVLIASFQYLQKCSY
 DEHAKLVQEVTDFAKTCVADESAANCDKSLHTLFGDKLCAIPNLRENYGELADCCTKQEP
 ERNECFLQHKDDNPSLPFERPEAEAMCTSFKENPTTFMGHYLHEVARRHPYFYAPELLY
 YAEQYNEILTQCCAEADKESCLTPKLDGVKEKALVSSVRQRMKCSSMQKFGERAFAKAWAV
 ARLSQTFPNADFAEITKLATDLTKVNKECCHGDLLECADDRAELAKYMCENQATISSKLQ
 TCCDKPLLKKAHCLSEVEHDTMPADLPAIAADFVEDQEVCKNYAEAKDVFLGTFLYEYSR
 RHPDYSVSLLLRLAKKYEATLEKCCAANPPACYGTVLAEFQPLVEEPKNLVKTNCDLYE
 KLGEYGFQNAILVRYTQKAPQVSTPTLVAAARNLGRVGTKCCTLPEDQRLPCVEDYLSAI
 LNRVCLLHEKTPVSEHVTKCCSGSLVERRPCFSALTVDETYVPKEFKAETFTFHSDICTL
 PEKEKQIKKQTALAEVLKHKPKATAEQLKTVMDFAQFLDTCCAADKDTCFSTEGPNLV
 TRCKDALA

Figure 5.8: Amino acid sequence of mouse serum albumin. The red highlights show two free cysteine residues.

5.5 Discussion

DDS is used for the treatment of leprosy because of its antibiotic and anti-inflammatory properties (Uetrecht et al. 1988; Zhu & Stiller 2001). *N*-acetyltransferase enzymes catalyse the conversion of DDS to stable mono and diacetylated derivatives, while cytochrome P-450, flavin monooxygenase and peroxidase-mediated *N*-hydroxylation results in the formation DDS hydroxylamine (Zuidema et al. 1986; Roychowdhury et al. 2007; Piyush M. Vyas et al. 2006). DDS hydroxylamine is susceptible to auto-oxidation and the derived nitroso species has been shown to bind covalently to protein (Roychowdhury et al. 2007; Bhaiya et al. 2006; Roychowdhury et al. 2005). Despite this, the nature of the binding interaction has not been defined. In order to characterize the antigenic structure that stimulated immune cells, *in-vitro* incubations of DDS-NO with MSA were performed. DDS-NO was found to covalently modify cysteine residue in MSA. In contrast to DDS-NO glutathione adducts, only one type of cysteine adduct in MSA was detected, corresponding to either a *N*-hydroxysulfinamide or sulfonamide adduct (described in chapter 2). This is consistent with previous findings that SMX-NO formed only one stable Cys34 adduct with human serum albumin. Notably, when DDS-NO was incubated with GSTP, all three types of adducts were detectable, though the sulfinamide and *N*-hydroxysulfonamide adducts were present at very low levels. Docking of DDS-NO to the binding sites on proteins may allow us to explain the difference observed between MSA and GSTP. Nonetheless, it is worth noting that there are two reactive cysteine residues on MSA, Cys34 and Cys579, however, modification was only detected on Cys34. It is possible that DDS-NO could modify both residues, however, the tryptic peptide containing modified Cys579 is too short to be detected by the current mass spectrometric method.

In this thesis to date, the immunogenicity of DDS in human systems was investigated. Immune responses resulting from DDS, SMX and their reactivate nitroso metabolites were also

evaluated using a mouse model. Analysis of DDS-NO protein adducts formed were performed based on previous studies on SMX-NO. Briefly, mice were immunized over a 2 weeks, dosing protocol after which, splenocytes were isolated and dose-dependent *in-vitro* proliferative responses and cross-reactivity measured. DDS-NO and SMX-NO were found to activate naïve T-cells during the 2 weeks dosing protocol. When isolated and restimulated, proliferative response were detected with nitroso compounds, but there was no cross reactivity with the parent drugs. Moreover, the parent drugs did not prime naive T-cells. These data are in stark contrast to the previous findings with human T-cells where DDS and DDS-NO both stimulated T-cells proliferate and secreted cytokines via different mechanisms. Furthermore, the results indicate that rodent models are viable models to study immunogenicity of haptenic compounds, but they fail to reproduce to direct binding of drugs to human MHC molecules.

Having successfully developed a model of DDS-NO in the mouse, the final section of the thesis focused on the assessment of DDS-NO modification of GSTpi and MSA using mass spectrometry.

In summary, our data indicate that DDS-NO can activate mouse naïve T-cells via a hapten pathway. DDS-NO formed three types of adducts with GSTpi and only one type of adduct in MSA. There are two limitations to the use of mouse model to evaluate the immunogenicity 1) Absence of human MHC restriction in the mouse which is an important risk factor of drug hypersensitivity. 2) The mouse does not form enough of the human drug reactive metabolite. For instance, shortage of sulfotransferase in the animal skin leads to lower level of the reactive sulfate metabolite of nevirapine (Sharma et al., 2013; Utrecht & Naisbitt 2013).

6. Final discussion

Clinical symptoms of drug hypersensitivity can vary from insignificant reactions such as nausea and skin rashes to severe conditions such as anaphylaxis, toxic epidermal necrolysis and liver failure. Drug hypersensitivity reactions have a bearing on patients, medical practitioners, and drug manufacturers. They limit therapeutic alternatives to treat ailments and sometimes cause fatalities (Schulkes et al., 2015). In England, adverse drug reactions cost the NHS approximately £ 470 million annually. Furthermore, the development of a new drug takes about ten years with an average cost of about \$ 6.3 billion per new drug. Consequently, the cost of removing a drug from the market is tremendous; hence, there is a need to predict and detect drug hypersensitivity at an early stage (Wu et al 2012). To do this requires an increased understanding of (1) patient susceptibility factors, (2) mechanisms of the drug-immune receptor interaction and (3) the nature of the immune cells responsible for initiating and propagating the reaction. In this thesis, we have focused on dapsone (DDS) hypersensitivity syndrome to explore each of these parameters.

DDS is a diaminophenyl sulphone, which has been efficaciously utilized in the treatment of leprosy for many years. The drug also has anti-inflammatory and antibacterial activity, and it is used to treat a variety of inflammatory skin ailments such as dermatitis herpetiformis, chronic bullous dermatoses. Common side of the drug includes nausea, dizziness, hepatitis, insomnia, and fatigue (Reibel et al., 2015). The authors indicated that about 1-4% of the people treated with the DDS experience a drug hypersensitivity syndrome. These reactions are could be mediated by either the parent drug or a protein reactive metabolite. DDS is metabolized to a hydroxylamine metabolite that undergoes spontaneous oxidation to nitroso DDS (Utrecht et al., 1988). Nitroso DDS may be detoxified through glutathione conjugation or bind to protein generating an adduct. It is hypothesized that formation of a DDS protein adduct is the molecular

initiating event in DDS hypersensitivity reactions. Thus, the primary objective of this research was to synthesize nitroso DDS as a reagent to compare its immunogenicity to the parent compound in human and murine systems.

Nitroso DDS was synthesized through oxidation of DDS hydroxylamine using iron chloride. The nitroso compound is insoluble in the reaction mixture and thus it was possible to separate it from the starting material in high purity through filtration. Similar to nitroso sulfamethoxazole, a highly immunogenic drug metabolite (Naisbitt et al., 1999; Farrell et al., 2003; Castrejon et al., 2010), nitroso DDS bound covalently to cysteine moieties on glutathione and model proteins. Currently, there are two hypotheses that describe the activation of T-cells with drugs. First, the PI concept states that drugs interact with either HLA molecules or HLA binding peptides directly through readily reversible bonds. Second, the hapten concept states that drugs bind covalently to non-MHC associated protein and that peptides liberated through protein processing (which presumably contain the drug metabolite) interact with HLA molecules. Both hypotheses are similar in that T-cells seem to require signals from (1) an HLA molecule, (2) an HLA binding peptide and the drug, for activation. Our data shows that nitroso DDS binds covalently to protein under cell culture conditions provides evidence that we have a reliable system to explore the immunogenicity of the parent drug and metabolite.

Our recently established *in vitro* priming assay can be used to explore the drug-specific stimulation of naïve T-cells from healthy donors (Faulkner et al. 2012). In this study, naïve T cells were primed in the presence of both DDS and nitroso DDS. The assay involved an initial co-culture period of naïve T-cells and dendritic cells with the drug or metabolite for two weeks. The primed T-cells were subsequently restimulated with a second batch of dendritic cells and both drugs to explore cross priming. Our study revealed that naïve T cells from HLA-B*13:01 negative donors were sensitized to DDS and nitroso DDS in a dose-dependent manner. Thus,

we next cloned individual T-cells from the priming assays to explore cellular phenotype and function.

Cytokines play a significant role in the immune reaction. They are soluble proteins that are secreted by cells of the immune system. The primary function of these cytokines is to alter the behaviour and properties of different cell types within and outside the immune system. Cytokine profiling is a fundamental parameter in understanding antigen-specific immune responses as it helps to define the behaviour of the various cells involved (O'Garra & Murphy 1994). There are many cytokine bioassays and these include; enzyme-linked immunosorbent assays (ELISAs), radioactive immunosorbent assays, microarrays and multiplex assays for detection of cytokines at the protein or mRNA level (Halmine et al., 1999). In regards to this study, we focused on an ELISpot method to profile the secretion of cytokines from drug-treated dendritic cells and T-cell clones. DDS and nitroso DDS-responsive clones isolated from the priming assay secreted IFN- γ and IL-13, but not IL-5 or the cytolytic molecule granzyme B. Results from these studies prompted us to investigate the cytokines secreted by cloned T-cells from patients with DDS hypersensitivity syndrome (see below).

To evaluate the role of APC in the stimulation of DDS-specific T-cell clones, clones were stimulated with DDS or nitroso DDS in presence or absence of irradiated autologous EBV transformed B-cells, which act as antigen presenting cells in the assay. Antigen presenting cells were found to be required for the activation of DDS-specific clones. To investigate further the mechanism of activation of DDS-specific clones, antigen presenting cell pulsing assays were performed. Pulsed APC failed to stimulate DDS-specific clones, which suggests that the drug is binding to HLA molecules in a labile manner. Furthermore, fixation of antigen presenting cells with glutaraldehyde, which blocks antigen processing, did not abolish activation of the DDS-specific T-cell clones. Thus, it is highly likely that clones were activated via a pharmacological interaction of the drug with immune receptors as originally proposed by

Pichler (2003). HLA molecules play a significant role in the T-cell response by providing an environment on the cell surface where peptides can be displayed to neighbouring cells (Pavlos et al., 2014). The DDS-specific clones all expressed the CD4+ cells surface protein and as expected the drug-specific response was restricted to MHC class II.

A limited number of nitroso DDS-responsive clones were generated from the healthy donor PBMC. Of these, only one CD8+ clones could be expanded in sufficient numbers for detailed analysis. In contrast to the DDS-responsive clones, the nitroso DDS responsive clone was activated via a hapten mechanism. Antigen presenting cells pulsed with nitroso DDS activated the clone, while fixation of antigen presenting cells significantly reduced the intensity of the response.

To investigate how these findings with PBMC and cloned T-cells compares to the response observed in hypersensitive patients, a new collaboration was initiated with clinical researchers in China. Six patients with DDS hypersensitivity syndrome were recruited. Patients presented with a range of symptoms including fever, skin rash and abnormal liver function tests. Importantly, all expressed the HLA risk allele HLA-B*13:01, which is strongly associated with the development of DDS hypersensitivity in the Chinese (Zhang et al., 2013). The lymphocyte transformation test, a diagnostic assay based on a comparison of levels of proliferation in drug- and medium-treated PBMC (Nyfeler and Pichler, 1997), and IFN- γ ELISpot were used to detect DDS and nitroso DDS-specific T-cell response in the patients. Patient PBMC were found to proliferate and secrete IFN- γ with both the parent drug and metabolite. Serial dilution was then used to isolate drug-responsive clones from the patients PBMC. DDS-specific and nitroso DDS-specific CD4+ clones with a stimulation index ranging from 2 to 120 were identified. Six of the DDS-specific clones showed a degree of cross-reactivity with nitroso DDS and/or the hydroxylamine metabolite. In contrast, clones were not activated with nitroso sulfamethoxazole. To determine whether DDS-specific clones cross reacted with closely

related sulphonamides and DDS analogues, we evaluated the reactivity of four DDS-specific clones to sulfamethoxazole, sulfamerazine, sulfadiazine, sulfanilamide, sulfadoxin, sulfachloropyridazine, 4, 4 diaminodiphenyl sulfide (DDT) and 3,3'-diaminodiphenyl sulfone (3DDS). 3DDS, which has high structural similarity to DDS, was found to activate clones; however, the other compounds yielded largely negative results. The nitroso DDS-specific clones showed no cross reactivity with DDS or nitroso sulfamethoxazole. Collectively, these data highlight the fine specificity of T-cells towards the structures of DDS and nitroso DDS. The pathways of activation of clones with DDS and nitroso DDS were the same as that observed with healthy volunteers. Namely, DDS-responsive clones were activated via a pi mechanism, whereas, nitroso DDS-responsive clones were activated via a hapten mechanism. With respect to cytokine secretion profiles, both DDS and DDS-NO specific secreted IFN- γ , IL-5, IL-13, and GB, IL-22 after drug treatment. The detection of granzyme B from the drug stimulated patient clones, but not with clones from healthy donors, indicates that the patient clones have a greater capacity to cause tissue injury.

Somewhat disappointedly, DDS- and nitroso DDS-responsive CD8⁺ clones were not detected in the cloning experiments with patient PBMC. These would have been interesting to study as the availability of antigen presenting cells from our frozen HLA-typed biobank containing PBMC from 1200 donors (Alfirevic et al., 2012) would have allowed us to explore whether DDS and/or nitroso DDS bind selectively to HLA-B*13:01 to activate T-cells. Importantly, in experiments conducted by other researchers in the Naisbitt research group since completion of my thesis, large numbers of CD8⁺ clones responsive towards both DDS and nitroso DDS have been generated through firstly purifying CD8⁺ T-cells prior to serial dilution. Initial studies suggest that both the parent drug and DDS-modified peptides selectively bind to HLA-B*13:01 to activate some of the CD8⁺ clones.

This study concluded by conducting a series of experiments using mice to explore the *in vivo* immunogenicity of DDS and nitroso DDS. Splenocytes were isolated from mice sensitized with DDS-NO and DDS, and *in vitro* proliferation assays were conducted to explore the extent of sensitization. Splenocytes from DDS-NO but not DDS sensitized mice were stimulated to proliferate *in vitro*, but only in the presence of the nitroso metabolite. These data add to the growing evidence that murine models can be used to explore the immunogenicity of drug haptens; however, they do not reproduce the T-cell responses to parent drugs that are seen in humans.

The detection of nitroso DDS-modified cysteine in mouse serum albumin by mass spectrometry revealed that a stable 2[O] adduct was formed. The cysteine adduct might be formed by two pathways: firstly, nucleophilic addition of cysteine thiolate to the aryl nitroso could result in a semimercaptal adduct, which can be further oxidized to a stable N-hydroxy sulfinamide; alternatively, cysteine could be oxidized to a cysteine sulfenic acid, followed by the nucleophilic addition to nitroso compound. The detection of the semimercaptal Cys47 adduct on GSTP suggests that the first pathway is highly possible. However, the general occurrence of protein sulphenic acids is also documented (Paulsen and Carroll, 2013). Sulphinic acids are well known to be soft nucleophiles due to their low pKa and can attack the nitrogen of the nitroso compounds to form an N-hydroxy sulphonamide.

In summary, our data indicates that nitroso DDS-NO can activate human and mouse naïve T-cells via a hapten pathway. In contrast, the parent drug only needs to bind reversibly to HLA molecules expressed on the surface of human antigen presenting cells to activate T-cells. It is important to emphasize that our healthy donors did not express the HLA risk allele HLA-B*13:01. Thus, both pathways of T-cell activation are feasible in donors expressing different HLA alleles. In on-going studies the Naisbitt group is exploring the nature of the drug-specific T-cell response and whether DDS and/or DDS-NO bind preferentially to HLA-B*13:01 to

selectively activate CD8⁺ T-cells. The data presented herein provides an initial understanding of the mechanisms of DDS hypersensitivity. A universal approach encompassing all the different aspects of drug hypersensitivity research will in the near future provide a complete and clear molecular pattern for understanding the pathogenesis of the reaction. We hope that sustained research will allow basic mechanistic observations to be translated into (1) the clinic to assist the development of tests that aid patient diagnosis and (2) Pharmaceutical industry through better drug design and synthesis.

Bibliography

Bibliography:

- Abdulahakeem Alsughayer, Abdel-Zaher A Elassar, Seham Mustafa, Fakhreia, Al Sagheer. (2011) "Synthesis, Structure Analysis and Antibacterial Activity of New Potent Sulfonamide Derivatives." J. Biomaterials and Nanobiotechnology **2**: 144
- Aberer, W., Bircher, A., Romano, A., Blanca, M., Campi, P., Fernandez, J., Brockow, K., Pichler, W. J. & Demoly, P. (2003). "Drug provocation testing in the diagnosis of drug hypersensitivity reactions: general considerations." Allergy **58**: 854-63.
- Adam J, Werner J, Pichler W, and Yerly D. (2011) "Delayed drug hypersensitivity: models of T-cell stimulation." Br J Clin Pharmacol **71**(5): 701–707.
- Agrawal, S. and A. Agarwalla. (2005). "Dapsone hypersensitivity syndrome: a clinico-epidemiological review." J Dermatol **32**(11): 883-9.
- AIDS. "and diagnostic laboratory immunology", **2**(2), 199-204.
- Akdis, C. A. & Akdis, M. (2009). "Mechanisms and treatment of allergic disease in the big picture of regulatory T cells." J Allergy Clin Immunol **123** :735-46.
- Alotaibi, M.H., Bahammam, S.A., ur Rahman, S., Bahnassy, A.A., Hassan, I.S., Alothman, A.F. and Alkayal, A.M., (2016). "The demographic and clinical characteristics of leprosy in Saudi Arabia." Journal of infection and public health **9**(5): 611-617.
- Alfirevic A, Gonzalez-Galarza F, Bell C, Martinsson K, Platt V, Bretland G, et al. (2012). In silico analysis of HLA associations with drug-induced liver injury: use of a HLA-genotyped DNA archive from healthy volunteers. Genome Med **4**(6): 51.
- Allday, E. and Barnes, J., 1951. Toxic effects of diaminodiphenyl-sulphone in treatment of leprosy. The Lancet, **258**(6675), pp.205-206.
- Alzahrani, A. et al., (2017). "Dapsone and Nitroso Dapsone Activation of Naïve T-Cells from Healthy Donors. Chemical Research in Toxicology", p.acs.chemrestox.7b00263.
- Anguizola, J., Matsuda, R., Barnaby, O. S., Hoy, K. S., Wa, C., DeBolt, E., and Hage, D. S., "2013". "Review: Glycation of human serum albumin." Clinica Chimica Acta **425**:64-76.
- Anguizola, J., Matsuda, R., Barnaby, O. S., Hoy, K. S., Wa, C., DeBolt, E., and Hage, D. S., "Antibody Conjugation". Bioconjugate Chemistry, **27**(10) : 2271-2275.
- Aucoin, D.P. et al., (1985). "Propylthiouracil-induced immune-mediated disease in the cat." J Pharmacol Exp Ther, **234**(1) : 13–18.
- Barranco, P. & Lopez-Serrano, M. C. (1998). "General and epidemiological aspects of allergic drug reactions." Clin Exp Allergy, **28** (Suppl 4): 61-2.

Bibliography

- Bates, D. W., Cullen, D. J., Laird, N., Petersen, L. A., Small, S. D., Servi, D., Laffel, G., Sweitzer, B. J., Shea, B. F., Hallisey, R. & Et Al. (1995). "Incidence of adverse drug events and potential adverse drug events. Implications for prevention. ADE Prevention Study Group." JAMA, **274**: 29-34.
- Beeler, A., Zaccaria, L., Kawabata, T., Gerber, B. O. & Pichler, W. J. (2008). "CD69 upregulation on T cells as an in vitro marker for delayed-type drug hypersensitivity." Allergy, **63**: 181-8.
- Belz, G. T., et al. (2005) "CD8 + Dendritic Cells Selectively Present MHC Class I-Restricted Noncytolytic Viral and Intracellular Bacterial Antigens In Vivo." The Journal of Immunology, **175** (1): 196-200.
- Ben M'Rad, M. et al., (2009). "Drug-induced hypersensitivity syndrome: Clinical and biologic disease patterns in 24 patients." Medicine, **88**: 131-140.
- Bendiner, E. (1981). "Baron von Pirquet: the aristocrat who discovered and defined allergy". Hosp Pract (Off Ed), **16**(137): 141-144.
- Bhaiya, P. et al., (2006). "Bioactivation, protein haptentation, and toxicity of sulfamethoxazole and dapsone in normal human dermal fibroblasts." Toxicology and applied pharmacology, **215**(2): 158-67.
- Bircher, A. J. & Scherer Hofmeier, K. (2012). "Drug hypersensitivity reactions: Inconsistency in the use of the classification of immediate and nonimmediate reactions". J Allergy Clin Immunol, **129**: 263-4.
- Blanca, M., Posadas, S., Torres, M. J., Leyva, L., Mayorga, C., Gonzalez, L., Juarez, C., Fernandez, J. & Santamaria, L. F. (2000). "Expression of the skin-homing receptor in peripheral blood lymphocytes from subjects with nonimmediate cutaneous allergic drug reactions." Allergy, **55**: 998-1004.
- Blaisdell, J., Goldstein, J.A., Munson, K.L., Hines, R.N. and Svensson, C.K., (2006). "Enzyme-mediated protein haptentation of dapsone and sulfamethoxazole in human keratinocytes: I. Expression and role of cytochromes P450." Journal of Pharmacology and Experimental Therapeutics, **319**(1): 488-496.
- Bluhm, R. E., Adedoyin, A., Mccarver, D. G. & Branch, R. A. (1999). "Development of dapsone toxicity in patients with inflammatory dermatoses: activity of acetylation and hydroxylation of dapsone as risk factors." Clin Pharmacol Ther, **65**: 598-605.
- Blum, Janice S., Pamela A. Wearsch, and Peter Cresswell.(2013) "Pathways of antigen processing." Annual review of immunology, **31**: 443-473.
- Boerma, J.S. et al., (2012). Mass spectrometric characterization of protein adducts of multiple P450-dependent reactive intermediates of diclofenac to human glutathione-S-transferase P1-1. Chemical Research in Toxicology, **25**(11): 2532-2541.

Bibliography

- Booth, S.A., Moody, C.E., Dahl, M.V., Herron, M.J. and Nelson, R.D., (1992). "Dapsone suppresses integrin-mediated neutrophil adherence function." Journal of investigative dermatology, **98**(2): 135-140.
- Bordin, L., Fiore, C., Zen, F., Coleman, M. D., Ragazzi, E. & Clari, G. (2010). "Dapsone hydroxylamine induces premature removal of human erythrocytes by membrane reorganization and antibody binding." Br J Pharmacol, **161**: 1186-99.
- Bos, J. D. & Kapsenberg, M. L. (1993). "The skin immune system: progress in cutaneous biology." Immunol Today, **14**: 75-8.
- Bourdi, M., Chen, W., Peter, R. M., Martin, J. L., Buters, J. T., Nelson, S. D. & Pohl, L. R. (1996). "Human cytochrome P450 2E1 is a major autoantigen associated with halothane hepatitis." Chem Res Toxicol, **9**: 1159-66.
- Bourdi, M., Larrey, D., Nataf, J., Bernuau, J., Pessayre, D., Iwasaki, M., Guengerich, F.P. and Beaune, P.H., (1990). "Anti-liver endoplasmic reticulum autoantibodies are directed against human cytochrome P-450IA2. A specific marker of dihydralazine-induced hepatitis." Journal of Clinical Investigation, **85**(6): 1967-1973
- Bouvy, J. C., De Bruin, M. L. & Koopmanschap, M. A. (2015). "Epidemiology of adverse drug reactions in Europe: a review of recent observational studies." Drug Saf, **38**: 437-53.
- Bozeman, P.M., Learn, D.B. and Thomas, E.L., (1992). "Inhibition of the human leukocyte enzymes myeloperoxidase and eosinophil peroxidase by dapsone." Biochemical pharmacology, **44**(3): 553-563.
- Brackett, C. C., Singh, H. & Block, J. H. (2004). "Likelihood and mechanisms of cross-allergenicity between sulfonamide antibiotics and other drugs containing a sulfonamide functional group." Pharmacotherapy, **24**: 856-70.
- Brander, C., Mauri-Hellweg, D., Bettens, F., Rolli, H., Goldman, M. and Pichler, W.J., (1995). "Heterogeneous T cell responses to beta-lactam-modified self-structures are observed in penicillin-allergic individuals." The Journal of immunology, **155**(5): 2670-2678.
- Britschgi, M., Steiner, U. C., Schmid, S., Depta, J. P., Senti, G., Bircher, A., Burkhart, C., Yawalkar, N. & Pichler, W. J. (2001). "T-cell involvement in drug-induced acute generalized exanthematous pustulosis." J Clin Invest, **107**: 1433-41.
- Brostoff, J., Scadding, G. K., Male, D., & Roitt, I. M. (1991). Introduction to Immune Responses. In J.Brostoff, G. K. Scadding, D. Male, & I. M. Roitt (Eds.), Clinical Immunology (New York: Gower Medical Publishing).
- Burkhart, C., Von Greyerz, S., Depta, J.P., Naisbitt, D.J., Britschgi, M., Park, K.B. and Pichler, W.J., (2001). "Influence of reduced glutathione on the proliferative response of sulfamethoxazole-specific and sulfamethoxazole-metabolite-specific human CD4+ T-cells." British journal of pharmacology, **132**(3): 623-630.

Bibliography

- Caddick, S. (2005). "Antibacterials and Antibiotics; Principle of Drug Design."
- Callan, H.E., Jenkins, R.E., Maggs, J.L., Levin, S.N., Clarke, S.E., Naisbitt, D.J. and Park, B.K., (2009). "Multiple adduction reactions of nitroso sulfamethoxazole with cysteinyl residues of peptides and proteins: implications for hapten formation." Chemical research in toxicology, **22**(5): 937-948.
- Cassell, G. H. & Mekalanos, J. (2001). "Development of antimicrobial agents in the era of new and reemerging infectious diseases and increasing antibiotic resistance." JAMA, **285**: 601-5.
- Castrejon, J.L., Berry, N., El-Ghaiesh, S., Gerber, B., Pichler, W.J., Park, B.K. and Naisbitt, D.J., (2010). "Stimulation of human T cells with sulfonamides and sulfonamide metabolites." Journal of Allergy and Clinical Immunology, **125**(2): 411-418.
- Caubet, J. C., Kaiser, L., Lemaitre, B., Fellay, B., Gervais, A. & Eigenmann, P. A. (2011). "The role of penicillin in benign skin rashes in childhood: a prospective study based on drug rechallenge." J Allergy Clin Immunol, **127**: 218-22.
- Chama, S., Prabhu, V.A., Doddapaneni, S., Singh, A., Pramoda, K., Thunga, G., Acharya, R., Bekur, R. and Dais, L., (2014). "Dapsone Hypersensitivity Syndrome in a Leprosy Patient." Journal of Pharmaceutical Research & Clinical Practice, **4**(2): 50-54.
- Cheng, L., Stewart, B.J., You, Q., Petersen, D.R., Ware, J.A., Piccotti, J.R., Kawabata, T.T. and Ju, C., (2008). "Covalent binding of the nitroso metabolite of sulfamethoxazole is important in induction of drug-specific T-cell responses in vivo." Molecular pharmacology, **73**(6): 1769-1775.
- Cheng, K.V., Arun, K. and Dutta, T.K., (2013). "Dapsone hypersensitivity syndrome: A rare life threatening complication of dapsone therapy." Journal of pharmacology & pharmacotherapeutics, **4**(2): 158.
- Cheng, C.Y., Su, S.C., Chen, C.H., Chen, W.L., Deng, S.T. and Chung, W.H., (2014). "HLA associations and clinical implications in T-cell mediated drug hypersensitivity reactions: an updated review." Journal of immunology research.
- Chen, Y.C. et al., (2013). "Drug reaction with eosinophilia and systemic symptoms: A drug-induced hypersensitivity syndrome with variable clinical features." Dermatologica Sinica, **31**(4): 196–204.
- Choquet-Kastylevsky, G., Vial, T. & Descotes, J. (2001). "Drug allergy diagnosis in humans: possibilities and pitfalls." Toxicology, **158**: 1-10.
- Christie, G., Coleman, J. W., Newby, S., Mcdiarmaid-Gordon, A., Hampson, J. P., Breckenridge, A. M. & Park, B. K. (1988). "A survey of the prevalence of penicillin-specific IgG, IgM and IgE antibodies detected by ELISA and defined by hapten inhibition, in patients with suspected penicillin allergy and in healthy volunteers." Br J Clin Pharmacol, **25**: 381-6.

Bibliography

- Chung, W. H., Hung, S. I., Yang, J. Y., Su, S. C., Huang, S. P., Wei, C. Y., Chin, S. W., Chiou, C. C., Chu, S. C., Ho, H. C., Yang, C. H., Lu, C. F., Wu, J. Y., Liao, Y. D. & Chen, Y. T. (2008). "Granulysin is a key mediator for disseminated keratinocyte death in Stevens-Johnson syndrome and toxic epidermal necrolysis". Nat Med,**14**: 1343-50.
- Chung, W.-H. et al., 2004. Medical genetics: A marker for Stevens–Johnson syndrome. Nature, **428**(6982), pp.486–486.
- Clarke JB, Maggs JL, Kitteringham NR, Park BK. (1990). "Immunogenicity of amodiaquine in the rat." Int Arch Allergy Appl Immunol,**91**:335–342.
- Classen, D. C., Pestotnik, S. L., Evans, R. S., Lloyd, J. F. & Burke, J. P. (1997). "Adverse drug events in hospitalized patients. Excess length of stay, extra costs, and attributable mortality." JAMA,**27**: 301-6.
- Coleman, M. D. (1993). "Dapsone: modes of action, toxicity and possible strategies for increasing patient tolerance." Br J Dermatol,**129**: 507-13.
- Coleman, M. D., Pahal, K. K. & Gardiner, J. M. (1996)." The effect of acetylation and deacetylation on the disposition of dapsone and monoacetyl dapsone hydroxylamines in human erythrocytes in-vitro." J Pharm Pharmacol,**48**: 401-6
- Coleman, M. D., Tingle, M.D., Hussain, F., Storr, R.C., & Park, B.K.(1991). "An investigation into the haematological toxicity of structural analogues of dapsone in-vivo and in-vitro." J Pharm Pharmacol,**43** (11): 779-84.
- Coombs, R., Gell, P. and Lachmann, P. (1975). Clinical aspects of immunology. Oxford: Blackwell Scientific Publications.
- Conforti, A., Costantini, D., Zanetti, F., Moretti, U., Grezzana, M. & Leone, R. (2012). "Adverse drug reactions in older patients: an Italian observational prospective hospital study." Drug Healthc Patient Saf,**4**: 75-80.
- Coopman, S. A., Johnson, R. A., Platt, R. & Stern, R. S. (1993). "Cutaneous disease and drug reactions in HIV infection." N Engl J Med,**328**: 1670-4.
- Cornejo-Garcia, J. A., Blanca-Lopez, N., Dona, I., Andreu, I., Agundez, J. A., Carballo, M., Blanca, M. & Canto, M. G. (2009). "Hypersensitivity reactions to non-steroidal anti-inflammatory drugs." Curr Drug Metab,**10**: 971-80.
- Costantino, C., Spooner, E., Ploegh, H. and Hafler, D. (2012). "Class II MHC Self-Antigen Presentation in Human B and T Lymphocytes." PLoS ONE,**7**(1): 29805.
- Crawford, L. A., & Weerapana, E., (2016). "A tyrosine-reactive irreversible inhibitor for glutathione S-transferase Pi (GSTP1)." Molecular BioSystems, **12**(6): 1768-1771.

Bibliography

- Criado, P. R., Criado, R. F. J., Avancini, J. D. M., & Santi, C. G. (2012). "Drug reaction with eosinophilia and systemic symptoms (DRESS)/drug-induced hypersensitivity syndrome (DIHS): a review of current concepts." Anais brasileiros de dermatologia, **87**(3): 435-449.
- Cribb, A. E. & Spielberg, S. P. (1992). "Sulfamethoxazole is metabolized to the hydroxylamine in humans." Clin Pharmacol Ther **51**: 522-6.
- Cribb, A. E., Spielberg, S. P. & Griffin, G. P. (1995). "N4-hydroxylation of sulfamethoxazole by cytochrome P450 of the cytochrome P4502C subfamily and reduction of sulfamethoxazole hydroxylamine in human and rat hepatic microsomes." Drug Metab Dispos, **23**: 406-14.
- Cribb, A.E. & Spielberg, S.P., 1990. An in vitro investigation of predisposition to sulphonamide idiosyncratic toxicity in dogs. Veterinary Research Communications, **14**(3), pp.241–252.
- Cribb, A.E. and Spielberg, S.P., (1990). "Hepatic microsomal metabolism of sulfamethoxazole to the hydroxylamine." Drug Metabolism and Disposition, **18**(5): 784-787.
- Cribb, A.E., Nuss, C.E., Alberts, D.W., Lamphere, D.B., Grant, D.M., Grossman, S.J. and Spielberg, S.P., (1996). "Covalent binding of sulfamethoxazole reactive metabolites to human and rat liver subcellular fractions assessed by immunochemical detection." Chemical research in toxicology, **9**(2): 500-507.
- Cribb, A.E., Spielberg, S.P. and Griffin, G.P., (1995). "N4-hydroxylation of sulfamethoxazole by cytochrome P450 of the cytochrome P4502C subfamily and reduction of sulfamethoxazole hydroxylamine in human and rat hepatic microsomes." Drug Metabolism and Disposition, **23**(3): 406-414.
- Cucinell, S. A., Israili, Z. H. & Dayton, P. G. (1972). "Microsomal N-oxidation of dapson a cause of methemoglobin formation in human red cells." Am J Trop Med Hyg, **21**: 322-31.
- Czerkinsky, C., Moldoveanu, Z., Mestecky, J., Nilsson, L. A. & Ouchterlony, O. (1988). "A novel two colour ELISPOT assay. I. Simultaneous detection of distinct types of antibody-secreting cells." J Immunol Methods, **115**: 31-7.
- Daftarian, M. P., Filion, L. G., Cameron, W., Conway, B., Roy, R., Tropper, F. & Diaz-Mitoma, F. (1995). "Immune response to sulfamethoxazole in patients with AIDS." Clin Diagn Lab Immunol, **2**: 199-204.
- David, E. G., Armen H. T., Ehrin J. A, and April W. A.(2011) "Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy," Lippincott Williams & Wilkins.
- Davies, E. C., Green, C. F., Taylor, S., Williamson, P. R., Mottram, D. R. & Pirmohamed, M. (2009). "Adverse drug reactions in hospital in-patients: a prospective analysis of 3695 patient-episodes." PLoS One, **4**: 4439.
- Daly, A.K. et al., 2009. HLA-B5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. Nature Genetics, **41**(7), pp.816–819

Bibliography

- De Haan, P., Bruynzeel, D. P. & Van Ketel, W. G. (1986). "Onset of penicillin rashes: relation between type of penicillin administered and type of immune reactivity". Allergy,**41**: 75-8.
- De Luca, L. & Giacomelli, G. (2008). "An easy microwave-assisted synthesis of sulfonamides directly from sulfonic acids." J Org Chem,**73**: 3967-9.
- Demoly, P., Adkinson, N. F., Brockow, K., Castells, M., Chiriac, A. M., Greenberger, P. A., & Sanchez-Borges, M. (2014). International consensus on drug allergy Allergy, **69**(4): 420-437.
- Den Haan, Joke M., et al.(2000) "Cd8+but Not Cd8–Dendritic Cells Cross-Prime Cytotoxic T Cells in Vivo." The Journal of Experimental Medicine,**192**(12): 1685-1696.
- Descamps, V. & Ranger-Rogez, S., 2014. DRESS syndrome. Joint Bone Spine, **81**(1), pp.15–21.
- Deshazo, R. D. & Kemp, S. F. (1997). "Allergic reactions to drugs and biologic agents." JAMA,**278**: 1895-906.
- Depta, J. P., F. Altnauer, et al. (2004). "Drug interaction with T-cell receptors: T-cell receptor density determines degree of cross-reactivity." J Allergy Clin Immunol,**113**(3): 519-527.
- Ding, H. X., Liu, K. K., Sakya, S. M., Flick, A. C. & O'donnell, C. J. (2013). "Synthetic approaches to the 2011 new drugs." Bioorg Med Chem,**21**: 2795-825.
- Dominguez, J. N., Leon, C., Rodrigues, J., Gamboa De Dominguez, N., Gut, J. & Rosenthal, P. J. (2005). "Synthesis and antimalarial activity of sulfonamide chalcone derivatives." Farmaco**60**: 307-11.
- Drayer, D. E., Strong, J. M., Jones, B., Sandler, A. & Reidenberg, M. M. (1974). "In vitro acetylation of drugs by human blood cells." Drug Metab Dispos,**2**: 499-505.
- Edwards, I. R. & Aronson, J. K. (2000). "Adverse drug reactions: definitions, diagnosis, and management." Lancet,**356**: 1255-9.
- Ellard, G.A. (1966). "Absorption, metabolism and excretion of di(p-aminophenyl) sulphone (dapsone) and di(p-aminophenyl) sulphoxide in man." Br j pharmacol chemother,**26**: 212–217.
- Elsheikh, A. et al., (2010). "Drug antigenicity, immunogenicity, and costimulatory signaling: evidence for formation of a functional antigen through immune cell metabolism." J. Immunol,**185**(11): 6448–60.
- Farrell, J. et al., (2003). "Characterization of sulfamethoxazole and sulfamethoxazole metabolite-specific T-cell responses in animals and humans." The Journal of pharmacology and experimental therapeutics, **306**(1): 229–37.

Bibliography

- Fattinger, K., Roos, M., Vergeres, P., Holenstein, C., Kind, B., Masche, U., Stocker, D. N., Braunschweig, S., Kullak-Ublick, G. A., Galeazzi, R. L., Follath, F., Gasser, T. & Meier, P. J. (2000). "Epidemiology of drug exposure and adverse drug reactions in two swiss departments of internal medicine." Br J Clin Pharmacol, **49**: 158-67.
- Faulkner, L., Martinsson, K., Santoyo-Castelazo, A., Cederbrant, K., Schuppe-Koistinen, I., Powell, H., Tugwood, J., Naisbitt, D.J. and Park, B.K., (2012). "The development of in vitro culture methods to characterize primary T-cell responses to drugs." Toxicological Sciences, **127**(1): 150-158.
- Faulkner, L., Gibson, A., Sullivan, A., Tailor, A., Usui, T., Alfirevic, A., Pirmohamed, M., Naisbitt, D.J. and Kevin Park, B., (2016). "Detection of primary T cell responses to drugs and chemicals in HLA-typed volunteers: implications for the prediction of drug immunogenicity". Toxicological Sciences, **154**(2): 416-429
- Fernandez, T. D., Canto, G. & Blanca, M. (2009). "Molecular mechanisms of maculopapular exanthema." Curr Opin Infect Dis, **22**: 272-8.
- Forna F., McConnell M., Kitabire F., Homsy J., Brooks J., Mermin J. & Weidle P. (2006) "Systematic review of the safety of trimethoprim-sulfamethoxazole for prophylaxis in HIV-infected pregnant women: implications for resource-limited settings." AIDS Rev, **8**(1):24-36.
- Fromm, E. & Wittmann, J., 1908. Derivate des p-Nitrothiophenols. Berichte der deutschen chemischen Gesellschaft, **41**(2), pp.2264–2273.
- Fujita T., Hansch C. (1967). "Analysis of the Structure-Activity Relationship of the Sulfonamide Drugs Using Substituent Constants." J. Med. Chem **10**: 991.
- Ganesan, S., Sahu, R., Walker, L. A. & Tekwani, B. L. (2010). "Cytochrome P450-dependent toxicity of dapsone in human erythrocytes." J Appl Toxicol, **30**: 271-5.
- Gelber, R., Peters, J. H., Gordon, G. R., Glazko, A. J. & Levy, L. (1971). "The polymorphic acetylation of dapsone in man." Clin Pharmacol Ther, **12**: 225-38
- Genc, Y., Ozkanca, R. & Bekdemir, Y. (2008). "Antimicrobial activity of some sulfonamide derivatives on clinical isolates of *Staphylococcus aureus*." Ann Clin Microbiol Antimicrob, **7**: 17
- Gill, H.J., Hough, S.J., Naisbitt, D.J., Maggs, J.L., Kitteringham, N.R., Pirmohamed, M. and Park, B.K., (1997). "The relationship between the disposition and immunogenicity of sulfamethoxazole in the rat." Journal of Pharmacology and Experimental Therapeutics, **282**(2): 795-801.
- Girard, P.M., (1993). "Dapsone—pyrimethamine compared with aerosolized pentamidine as primary prophylaxis against *Pneumocystis carinii* pneumonia and toxoplasmosis in HIV infection." N Engl J Med, **328**: 1514-20
- Godeau, B., Oksenhendler, E. & Bierling, P. (1993). "Dapsone for autoimmune thrombocytopenic purpura." Am J Hematol, **44**: 70-2.

Bibliography

- Gomes, E. R. & Demoly, P. (2005). "Epidemiology of hypersensitivity drug reactions." Curr Opin Allergy Clin Immunol, **5**: 309-16.
- Gorbachev, A. V. & Fairchild, R. L. (2001). "Induction and regulation of T-cell priming for contact hypersensitivity." Crit Rev Immunol, **21**: 451-72.
- Guglielmi, L., P. Guglielmi, et al. (2006). "Drug hypersensitivity: Epidemiology and risk factors." Curr Pharm Des, **12**(26): 3309-3312.
- Graul, A. I., Lupone, B., Cruces, E. & Stringer, M. (2013). "2012 in review - part I: the year's new drugs & biologics." Drugs Today (Barc), **49**: 33-68
- Greenberger, P. A. (2006). 8. "Drug allergy." J Allergy Clin Immunol, **117**: S464-70.
- Gruchalla, R. S. (2001). "Drug metabolism, danger signals, and drug-induced hypersensitivity." J Allergy Clin Immunol, **108**: 475-88.
- Gyllensten, H., Rehnberg, C., Jonsson, A. K., Petzold, M., Carlsten, A. & Andersson Sundell, K. (2013). "Cost of illness of patient-reported adverse drug events: a population-based cross-sectional survey." BMJ Open, **3**.
- Haddi, E., Charpin, D., Tafforeau, M., Kulling, G., Lanteaume, A., Kleisbauer, J. P. & Vervloet, D. (1990). "Atopy and systemic reactions to drugs." Allergy, **45**: 236-9.
- Hall, R. P., Lawley, T. J., Smith, H. R. & Katz, S. I. (1982). "Bullous eruption of systemic lupus erythematosus. Dramatic response to dapsone therapy." Ann Intern Med, **97**: 165-70
- Hallas, J., Harvald, B., Gram, L. F., Grodum, E., Brosen, K., Haghfelt, T. & Damsbo, N. (1990). "Drug related hospital admissions: the role of definitions and intensity of data collection, and the possibility of prevention." J Intern Med, **228**: 83-90.
- Halminen, M., M. Sjoroos, M. J. Makela, M. Waris, E. Terho, T. Lovgren, and J. Ilonen. (1999). "Simultaneous detection of IFN-gamma and IL-4 mRNAs using RT-PCR and time-resolved fluorometry." Cytokine, **11**: 87-93.
- Hargus, S. J., Amouzedeh, H. R., Pumford, N. R., Myers, T. G., McCoy, S. C. & Pohl, L. R. (1994). "Metabolic activation and immunochemical localization of liver protein adducts of the nonsteroidal anti-inflammatory drug diclofenac." Chem Res Toxicol, **7**: 575-82.
- Hansch, C., Sammes, P., Taylor, J., Kennewell, P., Emmett, J., Ramsden, C. and Drayton, C. (1990). Comprehensive medicinal chemistry. Oxford,: Pergamon Press.
- Hausmann, O. V., Gentinetta, T., Bridts, C. H. & Ebo, D. G. (2009). "The basophil activation test in immediate-type drug allergy." Immunol Allergy Clin North Am, **29**: 555-66.
- Hazell, L. & Shakir, S. A. (2006). "Under-reporting of adverse drug reactions : a systematic review." Drug Saf, **29**: 385-96.

Bibliography

- Heath, William R., and Francis R. Carbone. (2001) "cross-presentation, dendritic cells, tolerance and immunity." Annual Review of Immunology, **19**(1): 47-64.
- Hoffmann, E., et al. (2000) "A DNA transfection system for production of influenza A virus from eight plasmids." Proceedings of the National Academy of Sciences, **97**(11): 6108-6113.
- Hoogeveen, R. M., (2016). "Fatal dapsone hypersensitivity syndrome related eosinophilic myocarditis: case report." Reactions, **1594**:75-26.
- Houde, Mathieu, et al. (2003) "Phagosomes are competent organelles for antigen cross-presentation." Nature, **425**(6956): 402-406.
- Howard, R. L., Avery, A. J., Slavenburg, S., Royal, S., Pipe, G., Lucassen, P. & Pirmohamed, M. (2007). "Which drugs cause preventable admissions to hospital? A systematic review." Br J Clin Pharmacol, **63**: 136-47.
- Hung, S.I., Chung, W.H., Liou, L.B., Chu, C.C., Lin, M., Huang, H.P., Lin, Y.L., Lan, J.L., Yang, L.C., Hong, H.S. and Chen, M.J., (2005). "HLA-B* 5801 allele as a genetic marker for severe cutaneous adverse reactions caused by allopurinol. Proceedings of the National Academy of Sciences of the United States of America, **102**(11): 4134-4139.
- Humljan, J. & Gobec, S. (2005). "Synthesis of N-phthalimido β -aminoethanesulfonyl chlorides: the use of thionyl chloride for a simple and efficient synthesis of new peptidosulfonamide building blocks." Tetrahedron Letters, **46**: 4069-4072.
- Illing, P. T., Purcell, A. W., & McCluskey, J. (2017). "The role of HLA genes in pharmacogenomics: unravelling HLA associated adverse drug reactions." Immunogenetics, **69**(8–9), pp.617–630.
- Illing, P. T., Vivian, J. P., Dudek, N. L., Kostenko, L., Chen, Z., Bharadwaj, M., Miles, J. J., Kjer-Nielsen, L., Gras, S., Williamson, N. A., Burrows, S. R., Purcell, A. W., Rossjohn, J. & McCluskey, J. (2012). Immune self-reactivity triggered by drug-modified HLA-peptide repertoire. Nature, **486**: 554-8.
- Isik, S., Kockar, F., Aydin, M., Arslan, O., Guler, O. O., Innocenti, A., Scozzafava, A. & Supuran, C. T. (2009). "Carbonic anhydrase inhibitors: inhibition of the beta-class enzyme from the yeast *Saccharomyces cerevisiae* with sulfonamides and sulfamates." Bioorg Med Chem, **17**: 1158-63.
- Jancova, P. & Siller, M., 2012. Phase II Drug Metabolism. In *Topics on Drug Metabolism*. Available at: <http://www.intechopen.com/books/topics-on-drug-metabolism/phase-ii-drug-metabolism>.

Bibliography

- James, L. K., Shamji, M. H., Walker, S. M., Wilson, D. R., Wachholz, P. A., Francis, J. N., Jacobson, M. R., Kimber, I., Till, S. J. & Durham, S. R. (2011). "Long-term tolerance after allergen immunotherapy is accompanied by selective persistence of blocking antibodies." J Allergy Clin Immunol, **127**: 509-516.
- Jemal, A., Ward, E., Hao, Y. & Thun, M. (2005). "Trends in the leading causes of death in the United States, 1970-2002". JAMA, **294**: 1255-9.
- Jenkins, R.E., Kitteringham, N.R., Goldring, C.E., Dowdall, S.M., Hamlett, J., Lane, C.S., Boerma, J.S., Vermeulen, N.P. and Park, B.K., (2008). "{Glutathione-S-transferase pi as a model protein for the characterisation of chemically reactive metabolites." Proteomics, **8**(2): 301-315.
- Jenkinson, C. et al., (2009). "A mechanistic investigation into the irreversible protein binding and antigenicity of p-phenylenediamine." Chemical Research in Toxicology, **22**(6): 1172–1180.
- Jensen, P. (2007). "Recent advances in antigen processing and presentation." Nature Immunology, **8**(10): 1041-1048.
- Jick, H. (1984). "Adverse drug reactions: the magnitude of the problem." J Allergy Clin Immunol, **74**: 555-7.
- Johansson, S. G., Bieber, T., Dahl, R., Friedmann, P. S., Lanier, B. Q., Lockey, R. F., Motala, C., Ortega Martell, J. A., Platts-Mills, T. A., Ring, J., Thien, F., Van Cauwenberge, P. & Williams, H. C. (2004). "Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization". J Allergy Clin Immunol, **113**: 832-6.
- Johansson, S. G., Hourihane, J. O., Bousquet, J., Brujinzeel-Koomen, C., Dreborg, S., Haahtela, T., Kowalski, M. L., Mygind, N., Ring, J., Van Cauwenberge, P., Van Hage-Hamsten, M. & Wuthrich, B. (2001). "A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature task force." Allergy, **56**: 813-24.
- Jorde, U.P., Horowitz, H.W. and Wormser, G.P., (1993). "Utility of dapsone for prophylaxis of *Pneumocystis carinii* pneumonia in trimethoprim-sulfamethoxazole-intolerant, HIV-infected individuals." Aids, **7**(3): 355-360.
- Kalish, R. S. (1995). "Antigen processing: the gateway to the immune response." J Am Acad Dermatol, **32**: 640-52.
- K., Strateny, K. & Kristova, V. (2009). "Adverse drug reactions related to hospital admission in Slovak elderly patients." Arch Gerontol Geriatr, **48**: 186-90.
- Kansyl, M., Seydel, J. K., Wiese, M. & Haller, R. (1992). "Synthesis of new 2,4-diamino-5-benzylpyrimidines active against various bacterial species." European Journal of Medicinal Chemistry, **27**: 237-244.

Bibliography

- Karlin, E. and Phillips, E., (2014). "Genotyping for severe drug hypersensitivity." Current allergy and asthma reports**14**(3): 418.
- Knowles S, Shapiro L, Shear, NH. (2001). "Should celecoxib be contraindicated in patients who are allergic to sulfonamides? Revisiting the meaning of 'sulfa' allergy." Drug Safety,**24**:239-247
- Knowles, S. R., Uetrecht, J. & Shear, N. H. (2000). "Idiosyncratic drug reactions: the reactive metabolite syndromes." Lancet,**356**: 1587-91.
- Knowles, S. R., Uetrecht, J. P. & Shear, N. H. (2002). "Confirming false adverse reactions to drugs by performing individualized, randomized trials." Can J Clin Pharmacol,**9**: 149-53.
- Kim, S. H., Y. M. Ye, et al. (2010). "Genetic and ethnic risk factors associated with drug hypersensitivity." Curr Opin Allergy Clin Immunol,**10**(4): 280-290.
- Ko, T.M., Chung, W.H., Wei, C.Y., Shih, H.Y., Chen, J.K., Lin, C.H., Chen, Y.T. and Hung, S.I., (2011). "Shared and restricted T-cell receptor use is crucial for carbamazepine-induced Stevens-Johnson syndrome." Journal of Allergy and Clinical Immunology, **128**(6): 1266-1276.
- Kornbrust, D., Eydeloth, R., & Garratty, G. (1989). Investigations of the potential for five beta-lactam antibiotics to elicit type II hypersensitivity reactions in rats and monkeys. Fundam.Appl.Toxicol., **12**, 558-566.
- Kosseifi, S.G., Guha, B., Nassour, D.N., Chi, D.S. and Krishnaswamy, G., (2006). "The Dapsone hypersensitivity syndrome revisited: a potentially fatal multisystem disorder with prominent hepatopulmonary manifestations." Journal of Occupational Medicine and Toxicology,**1**(1): 9.
- Kovacsovics-Bankowski, M., and K. Rock. (1995) "A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules." Science,**267** (5195): 243-246.
- Kucera CM, Greenberger PA. (1996)." Adverse drug reactions: treatment and prevention." Hosp Med,**32**:11-24
- Kuechler, P. C., Britschgi, M., Schmid, S., Hari, Y., Grabscheid, B. & Pichler, W. J. (2004). "Cytotoxic mechanisms in different forms of T-cell-mediated drug allergies." Allergy,**59**: 613-22.
- Kundig, T., et al. (1995) "Fibroblasts as efficient antigen-presenting cells in lymphoid organs." Science**268**(5215): 1343-1347.
- Kurts, Christian, et al. (1997) "Class I-restricted Cross-Presentation of Exogenous Self-Antigens Leads to Deletion of Autoreactive CD8+T Cells." The Journal of Experimental Medicine, **186**(2): 239-245.

Bibliography

- Kurts, Christian, et al.(1998) "The Peripheral Deletion of Autoreactive CD8+T Cells Induced by Cross-presentation of Self-antigens Involves Signaling through CD95 (Fas, Apo-1)." The Journal of Experimental Medicine,**188**(2): 415-420.
- Landsteiner, K., & Jacobs, J. (1936). "Studies on the Sensitization of Animals With Simple Chemical Compounds Ii." The Journal of Experimental Medicine,**64**(10): 643–657.
- Landsverk, O., Bakke, O. and Gregers, T. (2009). "MHC II and the Endocytic Pathway: Regulation by Invariant Chain. Scandinavian Journal of Immunology, **70**(3): 184-193.
- Lange, U., Schumann, C., & Schmidt, K. L. (2001). "Current aspects of colchicine therapy--classical indications and new therapeutic uses." European journal of medical research, **6**(4): 150-160.
- Lanzavecchia, A. (2007). "Pillars article: Antigen-specific interaction between T and B cells." J Immunol**179**: 7206-8.
- Lavergne, S.N. and Trepanier, L.A., (2007). "Anti-platelet antibodies in a natural animal model of sulphonamide-associated thrombocytopaenia." Platelets, **18**(8): 595-604.
- Lavergne, S.N., Danhof, R.S., Volkman, E.M. and Trepanier, L.A., (2006). "Association of drug-serum protein adducts and anti-drug antibodies in dogs with sulphonamide hypersensitivity: A naturally occurring model of idiosyncratic drug toxicity." Clinical & Experimental Allergy **36**(7): 907-915.
- Lavergne, S.N., Park, B.K. and Naisbitt, D.J., (2008). "The roles of drug metabolism in the pathogenesis of T-cell-mediated drug hypersensitivity". Current opinion in allergy and clinical immunology, **8** (4): 299-307..
- Lazarou, J., Pomeranz, B. H. & Corey, P. N. (1998). "Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies." JAMA**279**: 1200-5.
- Lecoeur, S., Andre, C. & Beaune, P. H. (1996). "Tienilic acid-induced autoimmune hepatitis: anti-liver and-kidney microsomal type 2 autoantibodies recognize a three-site conformational epitope on cytochrome P450C9". Mol Pharmacol**50**: 326-33.
- Leeder, J. S., Riley, R. J., Cook, V. A. & Spielberg, S. P. (1992). "Human anti-cytochrome P450 antibodies in aromatic anticonvulsant-induced hypersensitivity reactions." J Pharmacol Exp Ther**263**: 360-7.
- Levin, D., Lagassé, H. D., Burch, E., Strome, S., Tan, S., Jiang, H., and Golding, B., (2017). "Modulating immunogenicity of factor IX by fusion to an immunoglobulin Fc domain: a study using a hemophilia B mouse model." Journal of Thrombosis and Haemostasis, **15**(4): 721-734.
- Leysen, J., Sabato, V., Verweij, M. M., De Knop, K. J., Bridts, C. H., De Clerck, L. S. & Ebo, D. G. (2011). "The basophil activation test in the diagnosis of immediate drug hypersensitivity." Expert Rev Clin Immunol**7**: 349-55.

Bibliography

- Li, J. & Uetrecht, J. P. (2010). "The danger hypothesis applied to idiosyncratic drug reactions." Handb Exp Pharmacol 493-509.
- Li, J. G. & Osgood, E. E. (1949). "A method for the rapid separation of leukocytes and nucleated erythrocytes from blood of marrow with a phytohemagglutinin from red beans (*Phaseolus vulgaris*)." Blood 4: 670-5.
- Lipmann, F. (1945). "Acetylation of sulfanilamide by liver homogenates and extracts." Journal of Biological Chemistry 160(1): 173-190.
- Liu, H., Wang, C., Chu, T., Rani, P. L., Yu, D., Fu, X. A., ... & Zhang, F. (2014). "Case report of two cases of fever, rash, and organ involvement during the treatment of leprosy." PLoS neglected tropical diseases, 8(8): e3130.
- Lo Conte, M. & Carroll, K.S., (2012)." Chemoselective ligation of sulfinic acids with aryl-nitroso compounds." Angewandte Chemie - International Edition 51(26): 6502–6505.
- Lowe, J. and Smith, M., 1949. The Chemotherapy of Leprosy in Nigeria, with an Appendix on Glandular Fever and Exfoliative Dermatitis precipitated by Sulfones. International journal of Leprosy, 17(3), pp.181-95.
- Lu, R. J., Tucker, J. A., Zinevitch, T., Kirichenko, O., Konoplev, V., Kuznetsova, S., Sviridov, S., Pickens, J., Tandel, S., Brahmachary, E., Yang, Y., Wang, J., Freel, S., Fisher, S., Sullivan, A., Zhou, J., Stanfield-Oakley, S., Greenberg, M., Bolognesi, D., Bray, B., Koszalka, B., Jeffs, P., Khasanov, A., Ma, Y. A., Jeffries, C., Liu, C., Proskurina, T., Zhu, T., CHucholowski, A., LI, R. & Sexton, C. (2007)." Design and synthesis of human immunodeficiency virus entry inhibitors: sulfonamide as an isostere for the alpha-ketoamide group." J Med Chem 50: 6535-44.
- Luque, I., Leyva, L., Jose Torres, M., Rosal, M., Mayorga, C., Segura, J. M., Blanca, M. & Juarez, C. (2001). "In vitro T-cell responses to beta-lactam drugs in immediate and nonimmediate allergic reactions." Allergy 56: 611-8.
- Luyendyk, J.P. et al., (2006). "Unique gene expression and hepatocellular injury in the lipopolysaccharide-ranitidine drug idiosyncrasy rat model: Comparison with famotidine." Toxicological Sciences, 90(2): 569–585.
- Lipmann, F. & Tuttle, L.C., 1945. The detection of activated carboxyl groups with hydroxylamine as interceptor. The Journal of biological chemistry, 161, p.415.
- Macy, E. (2004). "Multiple antibiotic allergy syndrome." Immunol Allergy Clin North Am 24(3): 533-543.
- Madden, S., Maggs, J. L., Park, B. K. (1996). "Bioactivation of carbamazepine in the rat in vivo: evidence for the formation of reactive arene oxide(s)." Drug Metab Dispos 24: 469 -479.

Bibliography

- Maggs, J.L., Naisbitt, D. J., Tetley, J. N., Pirmohamed, M., and Park, B. K. (2000). "Metabolism of lamotrigine to a reactive arene oxide intermediate." Chem Res Toxicol**13**: 1075–1081.
- Mahmud, R. et al., (1997). Structural basis for the haemotoxicity of dapsone: The importance of the sulphonyl group. Toxicology, **117**(1), pp.1–11.
- Manchanda, T. et al., 2002. Haptenation of sulfonamide reactive metabolites to cellular proteins. Molecular pharmacology, **62**(5), pp.1011–1026.
- Mak, A. and Uetrecht, J., (2015). The Combination of Anti-CTLA-4 and PD1–/–Mice Unmasks the Potential of Isoniazid and Nevirapine To Cause Liver Injury. "Chemical research in toxicology,"**28**(12): 2287-2291.
- Makarov, V., Riabova, O. B., Yuschenko, A., Urlyapova, N., Daudova, A., Zipfel, P. F. & Mollmann, U. (2006). "Synthesis and antileprosy activity of some dialkylthiocarbamates." J Antimicrob Chemother**57**: 1134-8.
- Mallal, S. et al., (2002). "Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir." Lancet**359**(9308): 727–732.
- Mallal, S. et al., 2008. HLA-B*5701 Screening for Hypersensitivity to Abacavir. New England Journal of Medicine, **358**(6), pp.568–579
- Maren, T. H. (1976). "Relations between structure and biological activity of sulfonamides." Annual review of pharmacology and toxicology, **16**(1): 309-327.
- Marensi, V., Yap, M., Ji, Y., Lin, C., Berthiaume, L., and Leslie, E. M., (2016). "Glutathione Transferase P1 (GSTP1) is Modified by Palmitate." The FASEB Journal **30**(1 Supplement):1196-5.
- Markova A, Duquesnoy R, Levis W. (2014). "T-cell plasticity of dapsone and DRESS." J Drugs Dermatol**13**(1):12-3.
- Martin, M., Wurpts, G., Ott, H., Baron, J. M., Erdmann, S., Merk, H. F. & Sachs, B. (2010). "In vitro detection and characterization of drug hypersensitivity using flow cytometry." Allergy, **65** 32-9.
- Matzinger, P. (1994). "Tolerance, danger, and the extended family." Annu Rev Immunol**12**: 991-1045.
- Matzinger, P. (2002). "The danger model: a renewed sense of self". Science, **296**: 301-5.
- Mauri-Hellweg, D., Bettens, F., Mauri, D., Brander, C., Hunziker, T. and Pichler, W.J., (1995). Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin, and carbamazepine. The Journal of Immunology, **155**(1): 462-472.

Bibliography

- Meissner, T., Li, A., Biswas, A., Lee, K., Liu, Y., Bayir, E., Iliopoulos, D., van den Elsen, P. and Kobayashi, K. (2010). "NLR family member NLRC5 is a transcriptional regulator of MHC class I genes." Proceedings of the National Academy of Sciences, **107**(31): 13794-13799.
- Meng, X., Lawrenson, A., Berry, N., Maggs, J., French, N., Back, D., Khoo, S., Naisbitt, D. and Park, K. (2014). "Abacavir forms novel cross-linking abacavir protein adducts in patients." Clinical and Translational Allergy, **4**(Suppl 3): 38
- Meng, X., Al-Attar, Z., Yaseen, F.S., Jenkins, R., Earnshaw, C., Whitaker, P., Peckham, D., French, N.S., Naisbitt, D.J. and Park, B.K., (2017). "Definition of the nature and hapten threshold of the β -lactam antigen required for T cell activation in vitro and in patients." The Journal of Immunology, **198**(11): 4217-4227.
- Mennicke, M., Zawodniak, A., Keller, M., Wilkens, L., Yawalkar, N., Stickel, F., Keogh, A., Inderbitzin, D., Candinas, D. & Pichler, W. J. (2009). "Fulminant liver failure after vancomycin in a sulfasalazine-induced DRESS syndrome: fatal recurrence after liver transplantation." Am J Transplant **9**: 2197-202.
- Metz, M. & Maurer, M. (2009). "Innate immunity and allergy in the skin." Curr Opin Immunol, **21**: 687-93.
- Minor, A. H. & Burnett, L. (1948). "A method for obtaining living leukocytes from human peripheral blood by acceleration of erythrocyte sedimentation." Blood **3**: 799-802.
- Mofenson, L.M. et al., 2009. Guidelines for the Prevention and Treatment of Opportunistic Infections among HIV-exposed and HIV-infected children. MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports, **58**(RR-11), pp.1–166.
- Monshi, M.M., Faulkner, L., Gibson, A., Jenkins, R.E., Farrell, J., Earnshaw, C.J., Alfirevic, A., Cederbrant, K., Daly, A.K., French, N. and Pirmohamed, M., (2013). "Human leukocyte antigen (HLA)-B* 57: 01-restricted activation of drug-specific T cells provides the immunological basis for flucloxacillin-induced liver injury." Hepatology **57**(2): 727-739.
- Moreno-diaz, H., Villalobos-Molina, R., Ortiz-Andrade, R., Diaz-Coutino, D., Medina-Franco, J. L., Webster, S. P., Binnie, M., Estrada-Soto, S., Ibarra-Barajas, M., Leon-Rivera, I. & Navarrete-Vazquez, G. (2008). "Antidiabetic activity of N-(6-substituted-1,3-benzothiazol-2-yl)benzenesulfonamides." Bioorg Med Chem Lett **18**: 2871-7.
- Naisbitt, D. J., Farrell, J., Wong, G., Depta, J. P., Dodd, C. C., Hopkins, J. E., Gibney, C. A., Chadwick, D. W., Pichler, W. J., Pirmohamed, M. & Park, B. K. (2003). "Characterization of drug-specific T cells in lamotrigine hypersensitivity." J Allergy Clin Immunol **111**: 1393-403.
- Naisbitt, D. J., Pirmohamed, M. & Park, B. K. (2007). "Immunological principles of T-cell-mediated adverse drug reactions in skin." Expert Opin Drug Safe **6**: 109-24.

Bibliography

- Naisbitt, D.J., Farrell, J., Gordon, S.F., Maggs, J.L., Burkhart, C., Pichler, W.J., Pirmohamed, M. and Park, B.K., (2002). "Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation." Molecular pharmacology **62**(3): 628-637.
- Naisbitt, D.J., Hough, S.J., Gill, H.J., Pirmohamed, M., Kitteringham, N.R. and Park, B.K., (1999). "Cellular disposition of sulphamethoxazole and its metabolites: implications for hypersensitivity." British journal of pharmacology **126**(6): 1393-1407.
- Naisbitt, D.J., O'Neill, P.M., Pirmohamed, M. and Park, B.K., (1996). "Synthesis and reactions of nitroso sulphamethoxazole with biological nucleophiles: implications for immune mediated toxicity." Bioorganic & Medicinal Chemistry Letters **6**(13): 1511-1516.
- Naisbitt, D.J., Vilar, F.J., Stalford, A.C., Wilkins, E.G., Pirmohamed, M. and Park, B.K., (2000). "Plasma cysteine deficiency and decreased reduction of nitrososulfamethoxazole with HIV infection". AIDS research and human retroviruses **16**(18): 1929-1938.
- Nakayama, S., Atsumi, R., Takakusa, H., Kobayashi, Y., Kurihara, A., Nagai, Y., Nakai, D. and Okazaki, O., (2009). "A zone classification system for risk assessment of idiosyncratic drug toxicity using daily dose and covalent binding." Drug Metabolism and Disposition **37**(9):1970-1977..
- Naranjo, C. A., Busto, U., Sellers, E. M., Sandor, P., Ruiz, I., Roberts, E. A., Janecek, E., Domecq, C. & Greenblatt, D. J. (1981). "A method for estimating the probability of adverse drug reactions." Clin Pharmacol Ther **30**: 239-45.
- Nassif, A., Bensussan, A., Boumsell, L., Deniaud, A., Moslehi, H., Wolkenstein, P., Bagot, M. and Roujeau, J.C.(2004). "Toxic epidermal necrolysis: effector cells are drug-specific cytotoxic T cells." Journal of Allergy and Clinical Immunology **114**(5): 1209-1215.
- Nath, K. and Gupta, S. (2015). Severe fatal form of dapsone hypersensitivity syndrome: a case report. International Journal of Contemporary Pediatrics, **2**(1), p.50.
- Neefjes, J., M Jongsma, M.L. & Paul, P., (2011). "Towards a systems understanding of MHC class I and MHC class II antigen presentation." Nature Reviews Immunology **11**(12): 823–836.
- Nassif A, Bensussan A, Boumsell L, Deniaud A, Moslehi H, Wolkenstein P, et al. (2004). Toxic epidermal necrolysis: Effector cells are drug-specific cytotoxic T cells. Journal of Allergy and Clinical Immunology **114**(5): 1209-1215.
- Neuman, M. G., Malkiewicz, I. M. & Shear, N. H. (2000). "A novel lymphocyte toxicity assay to assess drug hypersensitivity syndromes." Clin Biochem **33**: 517-24.
- Ng, W., Lobach, A.R., Zhu, X., Chen, X., Liu, F., Metushi, I.G., Sharma, A., Li, J., Cai, P., Ip, J. and Novalen, M., (2012). "Animal models of idiosyncratic drug reactions." Advances in pharmacology **63**: 81.

Bibliography

- Noack, M. and Miossec, P.,(2014). "Th17 and regulatory T cell balance in autoimmune and inflammatory diseases." Autoimmunity reviews**13**(6): 668-677.
- Nopora, Katrin, et al. (2012) "MHC Class I Cross-Presentation by Dendritic Cells Counteracts Viral Immune Evasion." Frontiers in Immunology**3**.
- Norcross, M.A., Luo, S., Lu, L., Boyne, M.T., Gomarteli, M., Rennels, A.D., Woodcock, J., Margulies, D.H., McMurtrey, C., Vernon, S. and Hildebrand, W.H., (2012). "Abacavir induces loading of novel self-peptides into HLA-B* 57: 01: an autoimmune model for HLA-associated drug hypersensitivity." AIDS (London, England)**26**(11): F21.
- Nowell, P. C. (1960). "Phytohemagglutinin: an initiator of mitosis in cultures of normal human leukocytes." Cancer Res**20**: 462-6.
- Nyfeler, B. & Pichler, W. J. (1997). "The lymphocyte transformation test for the diagnosis of drug allergy: sensitivity and specificity." Clin Exp Allergy**27**: 175-81.
- O'Garra, A. & Murphy, K., 1994. Role of cytokines in determining T-lymphocyte function. Current Opinion in Immunology, **6**(3), pp.458–466.
- Ogese, M.O., Jenkins, R.E., Maggs, J.L., Meng, X., Whitaker, P., Peckham, D., Faulkner, L., Park, B.K. and Naisbitt, D.J., (2015). "Characterization of peroxidases expressed in human antigen presenting cells and analysis of the covalent binding of nitroso sulfamethoxazole to myeloperoxidase." Chemical research in toxicology**28**(1): 144-154.
- Ostrov, D.A., Grant, B.J., Pompeu, Y.A., Sidney, J., Harndahl, M., Southwood, S., Oseroff, C., Lu, S., Jakoncic, J., de Oliveira, C.A.F. and Yang, L., (2012). "Drug hypersensitivity caused by alteration of the MHC-presented self-peptide repertoire." Proceedings of the National Academy of Sciences, **109**(25): 9959-9964..
- Ozbek, N., Katircioglu, H., Karacan, N. & Baykal, T. (2007). "Synthesis, characterization and antimicrobial activity of new aliphatic sulfonamide." Bioorg Med Chem**15**: 5105-9. p. 1591-8.
- Paulsen, C. and Carroll, K. (2013). Cysteine-Mediated Redox Signaling: Chemistry, Biology, and Tools for Discovery. Chemical Reviews, **113**(7), pp.4633-4679.
- Padial, A., Antunez, C., Blanca-Lopez, N., Fernandez, T. D., Cornejo-Garcia, J. A., Mayorga, C., Torres, M. J. & Blanca, M. (2008). "Non-immediate reactions to beta-lactams: diagnostic value of skin testing and drug provocation test." Clin Exp Allergy**38**: 822-8.
- Padovan, E., Bauer, T., Tongio, M.M., Kalbacher, H. and Weltzien, H.U., (1997). "Penicilloyl peptides are recognized as T cell antigenic determinants in penicillin allergy." European journal of immunology**27**(6): 1303-1307.
- Pavlos, R., Mallal, S., Ostrov, D., Pompeu, Y. and Phillips, E., 2014. Fever, rash, and systemic symptoms: understanding the role of virus and HLA in severe cutaneous drug allergy. The Journal of Allergy and Clinical Immunology: In Practice, **2**(1), pp.21-33.

Bibliography

- Park, B. K. & Coleman, J. W. (1988). "The immunological basis of adverse drug reactions. A report on a symposium held in Liverpool on 6th April 1988." Br J Clin Pharmacol**26**, 491-5.
- Park, B. K., Coleman, J. W. & Kitteringham, N. R. (1987). "Drug disposition and drug hypersensitivity." Biochem Pharmacol**36**: 581-90.
- Park, B. K., Pirmohamed, M. & Kitteringham, N. R. (1998). "Role of drug disposition in drug hypersensitivity: a chemical, molecular, and clinical perspective." Chem Res Toxicol**11**: 969-88.
- Patel, K. J., Kedia, M. S., Bajpai, D., Mehta, S. S., Kshirsagar, N. A. & Gogtay, N. J. (2007). "Evaluation of the prevalence and economic burden of adverse drug reactions presenting to the medical emergency department of a tertiary referral centre: a prospective study." BMC Clin Pharmacol**7**: 8.
- Patrick, G.L., 2009. An Introduction to Medicinal Chemistry. UCL lecture notes, **40**, p.752.
- Patterson, J. T., Wilson, H. D., Asano, S., Nilchan, N., Fuller, R. P., Roush, W. R., and Barbas III, C. F., (2016). "Human Serum Albumin Domain I Fusion Protein for Antibody Conjugation." Bioconjugate Chemistry, **27**(10): 2271-2275
- Pavlos, R., Mallal, S., Ostrov, D., Pompeu, Y. and Phillips, E., (2014). "Fever, rash, and systemic symptoms: understanding the role of virus and HLA in severe cutaneous drug allergy." The Journal of Allergy and Clinical Immunology: In Practice **2**(1): 21-33.
- Phillips, E. and Mallal, S. (2018). Active suppression rather than ignorance: tolerance to abacavir-induced HLA-B*57:01 peptide repertoire alteration. Journal of Clinical Investigation.
- Phillips, E. J., Sullivan, J. R., Knowles, S. R. & Shear, N. H. (2002). "Utility of patch testing in patients with hypersensitivity syndromes associated with abacavir." AIDS**16**: 2223-5.
- Pichler, W. J. & Tilch, J. (2004). "The lymphocyte transformation test in the diagnosis of drug hypersensitivity." Allergy**59**: 809-20.
- Pichler, W. J. (2003). "Delayed drug hypersensitivity reactions." Ann Intern Med**139**: 683-93.
- Pichler, W. J., Schnyder, B., Zanni, M. P., Hari, Y. & Von Greysen, S. (1998). "Role of T cells in drug allergies." Allergy**53**: 225-32.
- Pichler, W.J., (2001). "Influence of reduced glutathione on the proliferative response of sulfamethoxazole-specific and sulfamethoxazole-metabolite-specific human CD4+ T-cells." British journal of pharmacology**132**(3): 623-630.
- Pichler, W.J., Adam, J., Watkins, S., Willemin, N., Yun, J. and Yerly, D., (2015). "Drug hypersensitivity: how drugs stimulate T cells via pharmacological interaction with immune receptors." International archives of allergy and immunology**168**(1): 13-24.

Bibliography

- Pirmohamed, M., Breckenridge, A. M., Kitteringham, N. R. & Park, B. K. (1998). "Adverse drug reactions." BMJ**316**: 1295-8.
- Pirmohamed, M., Graham, A., Roberts, P., Smith, D., Chadwick, D., Breckenridge, A. M. & Park, B. K. (1991). "Carbamazepine-hypersensitivity: assessment of clinical and in vitro chemical cross-reactivity with phenytoin and oxcarbazepine." Br J Clin Pharmacol**32**: 741-9.
- Pirmohamed, M., Naisbitt, D. J., Gordon, F. & Park, B. K. (2002). "The danger hypothesis--potential role in idiosyncratic drug reactions." Toxicology**181**: 55-63.
- Porebski, G., Gschwend-Zawodniak, A. & Pichler, W. J. (2011). "In vitro diagnosis of T cell-mediated drug allergy." Clin Exp Allergy**41**: 461-70.
- Posadas, S. J. & Pichler, W. J. (2007). "Delayed drug hypersensitivity reactions - new concepts." Clin Exp Allergy**37**: 989-99.
- Pouyanne, P., Haramburu, F., Imbs, J. L. & Begaud, B. (2000). "Admissions to hospital caused by adverse drug reactions: cross sectional incidence study. French Pharmacovigilance Centres." BMJ**320**: 1036.
- Powell, H., & Park, B. K. (2012). "The development of in vitro culture methods to characterize primary T-cell responses to drugs." Toxicological Sciences**127**(1): 150-158.
- Primeau, M. N. & Adkinson, N. F., Jr. (2001). "Recent advances in the diagnosis of drug allergy." Curr Opin Allergy Clin Immunol**1**: 337-41.
- Prussick, R. and Shear, N.H., (1996). "Dapsone hypersensitivity syndrome." Journal of the American Academy of Dermatology**35**(2): 346-349.
- Prussick, R., Ali, M.A., Rosenthal, D. and Guyatt, G., (1992). "The protective effect of vitamin E on the hemolysis associated with dapsone treatment in patients with dermatitis herpetiformis." Archives of dermatology **128**(2): 210-213.
- Revankar, G., Hanna, N., Imamura, N., Lewis, A., Larson, S., Finch, R., Avery, T. and Robins, R. (1990). ChemInform Abstract: Synthesis and in vivo Antitumor Activity of 2-Amino-9H-purine-6-sulfenamide, -sulfinamide, and -sulfonamide and Related Purine Ribonucleosides. *ChemInform*, **21**(22).
- Radice, A. & Sinico, R. A. (2005). "Antineutrophil cytoplasmic antibodies (ANCA)." Autoimmunity**38**: 93-103.
- Ramirez-Gonzalez, M. D., Herrera-Enriquez, M., Villanueva-Rodriguez, L. G. & Castell-Rodriguez, A. E. (2009). "Role of epidermal dendritic cells in drug-induced cutaneous adverse reactions." Handb Exp Pharmacol 137-62.
- Raschetti, R., Morgutti, M., Menniti-Ippolito, F., Belisari, A., Rossignoli, A., Longhini, P. & La Guidara, C. (1999). "Suspected adverse drug events requiring emergency department visits or hospital admissions." Eur J Clin Pharmacol**54**: 959-63.

Bibliography

- Rawlins, M. D. (1981). "Clinical pharmacology. Adverse reactions to drugs." Br Med J (Clin Res Ed)**282**: 974-6.
- Reantragoon, R., L. Kjer-Nielsen, et al. (2012). "Structural insight into MR1-mediated recognition of the mucosal associated invariant T cell receptor." Journal of Experimental Medicine **209**(4): 761-774.
- Reibel, F., Cambau, E. and Aubry, A., (2015). "Update on the epidemiology, diagnosis, and treatment of leprosy." Medecine et maladies infectieuses**45**(9): 383-393.
- Reid, M. J., Lockey, R. F., Turkeltaub, P. C. & Platts-Mills, T. A. (1993). "Survey of fatalities from skin testing and immunotherapy 1985-1989." J Allergy Clin Immunol**92**: 6-15.
- Renaudin, J.M., Beaudouin, E., Ponvert, C., Demoly, P. and Moneret-Vautrin, D.A., (2013). "Severe drug-induced anaphylaxis: analysis of 333 cases recorded by the Allergy Vigilance Network from 2002 to 2010." Allergy, **68**(7): 929-937.
- Revankar, G. R., Hanna, N. B., Ramasamy, K., Larson, S. B., Smee, D. F., Finch, R. A., Avery, T. L. & Robins, R. K. (1990). "Synthesis and In Vivo antitumor and antiviral activities of 2'-deoxyribofuranosyl and arabinofuranosyl nucleosides of certain purine-6-sulfenamides, sulfinamides and sulfonamides." Journal of Heterocyclic Chemistry**27**: 909-918.
- Rieder, M. J. (1994). "Mechanisms of unpredictable adverse drug reactions." Drug Saf**11**: 196-212.
- Rieder, M.J., Uetrecht, J., Shear, N.H. and Spielberg, S.P., (1988). "Synthesis and in vitro toxicity of hydroxylamine metabolites of sulfonamides." Journal of Pharmacology and Experimental Therapeutics **244**(2): 724-728.
- Riedl, M. A. & Casillas, A. M. (2003). "Adverse drug reactions: types and treatment options." Am Fam Physician**68**: 1781-90.
- Rietschel, R. L. (1996). "Reproducibility of patch-test results." Lancet**347**: 1202.
- Rigas, D. A. & Osgood, E. E. (1955). "Purification and properties of the phytohemagglutinin of *Phaseolus vulgaris*." J Biol Chem**212**: 607-15.
- Ring, J. & Brockow, K. (2002). "Adverse drug reactions: mechanisms and assessment." Eur Surg Res**34**: 170-5.
- Rizvi, S. and Raghavan, M. (2010). "Mechanisms of Function of Tapasin, a Critical Major Histocompatibility Complex Class I Assembly Factor". Traffic**11**(3): 332-347.
- Rodriguez-Monguio, R., Otero, M. J. & Rovira, J. (2003). "Assessing the economic impact of adverse drug effects." Pharmacoeconomics**21**: 623-50.

Bibliography

- Romano, A. & Demoly, P. (2007). "Recent advances in the diagnosis of drug allergy." Curr Opin Allergy Clin Immunol **7**: 299-303.
- Romano, A., Blanca, M., Torres, M. J., Bircher, A., Aberer, W., Brockow, K., Pichler, W. J. & Demoly, P. (2004). "Diagnosis of nonimmediate reactions to beta-lactam antibiotics." Allergy **59**: 1153-60.
- Romano, A., Torres, M. J., Castells, M., Sanz, M. L. & Blanca, M. (2011). "Diagnosis and management of drug hypersensitivity reactions." J Allergy Clin Immunol **127**: S67-73.
- Roujeau, J.C., 2013. Epidermal necrolysis (Stevens-Johnson syndrome and toxic epidermal necrolysis): Historical considerations. Dermatologica Sinica, **31**(4), pp.169–174.
- Rossi, R., Granoff, D. M., and Beernink, P. T., (2013). "Meningococcal factor H-binding protein vaccines with decreased binding to human complement factor H have enhanced immunogenicity in human factor H transgenic mice." Vaccine **31**(46): 5451-5457.
- Roush, W. R., Gwaltney, S. L., Cheng, J., Scheidt, K. A., Mckerrow, J. H. & Hansell, E. (1998). "Vinyl Sulfonate Esters and Vinyl Sulfonamides: Potent, Irreversible Inhibitors of Cysteine Proteases." Journal of the American Chemical Society **120**: 10994-10995.
- Roychowdhury, S., Vyas, P.M. and Svensson, C.K., (2007). "Formation and uptake of arylhydroxylamine-haptenated proteins in human dendritic cells." Drug Metabolism and Disposition **35**(4): 676-681.
- Roychowdhury, S., Vyas, P.M., Reilly, T.P., Gaspari, A.A. and Svensson, C.K., (2005). Characterization of the formation and localization of sulfamethoxazole and dapsone-associated drug-protein adducts in human epidermal keratinocytes. Journal of Pharmacology and Experimental Therapeutics **314**(1): 43-52.
- Rozieres, A., Hennino, A., Rodet, K., Gutowski, M. C., Gunera-Saad, N., Berard, F., Cozon, G., Bienvenu, J. & Nicolas, J. F. (2009a). "Detection and quantification of drug-specific T cells in penicillin allergy." Allergy **64**: 534-42.
- Rozieres, A., Vocanson, M., Said, B. B., Nosbaum, A. & Nicolas, J. F. (2009b). "Role of T cells in nonimmediate allergic drug reactions." Curr Opin Allergy Clin Immunol **9**: 305-10.
- Saeki, H., Moore, A. M., Brown, M. J., & Hwang, S. T. (1999). "Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes." Journal of Immunology **162**(5): 2472–5.
- Sago, J and Hall R. (2002). "Dapsone". Dermatologic Therapy **15**: 340-351
- Salama, A. D., Levy, J. B., Lightstone, L. & Pusey, C. D. (2001). "Goodpasture's disease". Lancet **358**: 917-20.

Bibliography

- Sallusto, F., Schaerli, P., Loetscher, P., Schaniel, C., Lenig, D., Mackay, C. R., Lanzavecchia, A. (1998). "Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation." *European Journal of Immunology*, **28**(9): 2760–2769.
- Sampson, H. A. & Albergo, R. (1984). "Comparison of results of skin tests, RAST, and double-blind, placebo-controlled food challenges in children with atopic dermatitis." *J Allergy Clin Immunol* **74**: 26-33.
- Sánchez-Gómez, F., Díez-Dacal, B., García-Martín, E., Agúndez, J., Pajares, M. and Pérez-Sala, D. (2016). "Detoxifying Enzymes at the Cross-Roads of Inflammation, Oxidative Stress, and Drug Hypersensitivity: Role of Glutathione Transferase P1-1 and Aldose Reductase." *Frontiers in Pharmacology* **7**.
- Sarich, T.C. et al., (1995). "A model of isoniazid-induced hepatotoxicity in rabbits." *Journal of Pharmacological and Toxicological Methods* **34**(2): 109–116.
- Satoguina, J. S., Adjobimey, T., Arndts, K., Hoch, J., Oldenburg, J., Layland, L. E. & Hoerauf, A. (2008). "Tr1 and naturally occurring regulatory T cells induce IgG4 in B cells through GITR/GITR-L interaction, IL-10 and TGF-beta." *Eur J Immunol* **38**: 3101-13.
- Schlapbach, C., Zawodniak, A., Irla, N., Adam, J., Hunger, R. E., Yerly, D., Pichler, W. J. & Yawalkar, N. (2011). "NKp46+ cells express granulysin in multiple cutaneous adverse drug reactions." *Allergy* **66**: 1469-76.
- Schnyder, B., Burkhart, C., Schnyder-Frutig, K., von Greyerz, S., Naisbitt, D.J., Pirmohamed, M., Park, B.K. and Pichler, W.J., (2000). "Recognition of sulfamethoxazole and its reactive metabolites by drug-specific CD4+ T cells from allergic individuals." *The Journal of Immunology* **164**(12): 6647-6654
- Schnyder, B., Mauri-Hellweg, D., Zanni, M., Bettens, F. and Pichler, W.J., (1997). "Direct, MHC- dependent presentation of the drug sulfamethoxazole to human alphabeta T cell clones." *Journal of Clinical Investigation* **100**(1): 136-141.
- Schuerwegh, A. J., Van Offel, J. F., Stevens, W. J., Bridts, C. H. & De Clerck, L. S. (2003). "Influence of therapy with chimeric monoclonal tumour necrosis factor-alpha antibodies on intracellular cytokine profiles of T lymphocytes and monocytes in rheumatoid arthritis patients." *Rheumatology* **42**: 541-8.
- Schulkes, K.J., Tervaert, J.C., Rijken, F. and Haas, L.E., (2015). "Dapsone hypersensitivity syndrome not related to G6PD deficiency." *BMJ case reports*.
- Schmid, D. A., J. P. H. Depta, et al. (2006). "Transfection of drug-specific T-cell receptors into hybridoma cells: Tools to monitor drug interaction with T-cell receptors and evaluate cross-reactivity to related compounds." *Mol Pharmacol* **70**(1): 356-365.
- Scott, H., Fausa, O., Ek, J., Valnes, K., Blystad, L. & Brandtzaeg, P. (1990). "Measurements of serum IgA and IgG activities to dietary antigens. A prospective study of the diagnostic usefulness in adult coeliac disease." *Scand J Gastroenterol* **25**: 287-92.
- Sebastiani, S., Albanesi, C., De, P. O., Puddu, P., Cavani, A. & Girolomoni, G. (2002). "The role of chemokines in allergic contact dermatitis." *Arch Dermatol Res* **293**: 552-9.

Bibliography

- Seydel, J. K. (1968). "Sulfonamides, structure-activity relationship, and mode of action. Structural problems of the antibacterial action of 4-aminobenzoic acid (PABA) antagonists." Journal of pharmaceutical sciences **57**(9): 1455-1478.
- Shah, S. S., Rivera, G. & Ashfaq, M. (2013). "Recent advances in medicinal chemistry of sulfonamides. Rational design as anti-tumoral, anti-bacterial and anti-inflammatory agents." Mini Rev Med Chem **13**: 70-86.
- Shanks, G.D., Edstein, M.D., Suriyamongkol, V., Timsaad, S. and Webster, H.K., (1992). "Malaria chemoprophylaxis using proguanil/dapsone combinations on the Thai-Cambodian border." The American journal of tropical medicine and hygiene, **46**(6): 643-648..
- Sharma, A.M. & Uetrecht, J., (2014). "Bioactivation of drugs in the skin: relationship to cutaneous adverse drug reactions." Drug Metabolism Reviews **46**(1): 1–18
- Shear, N. H. & Spielberg, S. P. (1988). "Anticonvulsant hypersensitivity syndrome. In vitro assessment of risk." J Clin Invest **82**: 1826-32.
- Shear, N. H., Spielberg, S. P., Grant, D. M., Tang, B. K. & Kalow, W. (1986). "Differences in metabolism of sulfonamides predisposing to idiosyncratic toxicity." Ann Intern Med **105**: 179-84.
- Shekhar, S., Dunn, T. B., Kotecki, B. J., Montavon, D. K. & Cullen, S. C. (2011). "A general method for palladium-catalyzed reactions of primary sulfonamides with aryl nonaflates." J Org Chem **76**: 4552-63
- Shenton, J.M. et al., 2003. Characterization of a potential animal model of an idiosyncratic drug reaction: Nevirapine-induced skin rash in the rat. Chemical Research in Toxicology, **16**(9), pp.1078–1089.
- Shepherd, G., Mohorn, P., Yacoub, K. & May, D. W. (2012). "Adverse drug reaction deaths reported in United States vital statistics, 1999-2006." Ann Pharmacother **46**: 169-75.
- Shiohara, T., M. Inaoka, et al. (2006). "Drug-induced hypersensitivity syndrome (DIHS): a reaction induced by a complex interplay among herpesviruses and antiviral and antidrug immune responses." Allergol Int **55**(1): 1-8.
- Sikdar, K. C., Dowden, J., Alaghebandan, R., Macdonald, D., Peter, P. & Gadag, V. (2012). "Adverse drug reactions in elderly hospitalized patients: a 12-year population-based retrospective cohort study." Ann Pharmacother **46**: 960-71.
- Sinari, S., Nedelkov, D., Reaven, P., and Billheimer, D., (2017). "The Analysis of Human Serum Albumin Proteoforms Using Compositional Framework. In Statistical Analysis of Proteomics, Metabolomics, and Lipidomics Data Using Mass Spectrometry" 141-155. Springer International Publishing.
- Singh, I. K., Srinivas, S., and Tripathi, B. K., (2016). "An unusual presentation of dapsone hypersensitivity syndrome." Contains 84 pages from 85 to 164 (inclusive of all advertisements), **17**(2): 123.

Bibliography

- Smith, C. C., Bennett, P. M., Pearce, H. M., Harrison, P. I., Reynolds, D. J., Aronson, J. K. & Grahame-Smith, D. G. (1996). "Adverse drug reactions in a hospital general medical unit meriting notification to the Committee on Safety of Medicines." Br J Clin Pharmacol**42**: 423-9.
- Smith, T. F. (1992). "Allergy testing in clinical practice." Ann Allergy**68**: 293-301.
- Spielberg, S. P. (1980). "Acetaminophen toxicity in human lymphocytes in vitro." J Pharmacol Exp Ther**213**, 395-8.
- Spielberg, S. P. (1984). "In vitro assessment of pharmacogenetic susceptibility to toxic drug metabolites in humans". Fed Proc**43**: 2308-13.
- Spielberg, S. P. (1986). "In vitro analysis of idiosyncratic drug reactions". Clin Biochem**19**: 142-4.
- Stacey, J. W. (1943). "The inhalation of nebulized solutions of sulfonamides in the treatment of bronchiectasis." CHEST Journal**9**(4): 302-306.
- Stapel, S. O., Asero, R., Ballmer-Weber, B. K., Knol, E. F., Strobel, S., Vieths, S. & Kleine-Tebbe, J. (2008). "Testing for IgG4 against foods is not recommended as a diagnostic tool: EAACI Task Force Report." Allergy**63**: 793-6.
- Supuran, C. T., Casini, A. & Scozzafava, A. (2003). "Protease inhibitors of the sulfonamide type: anticancer, antiinflammatory, and antiviral agents." Med Res Rev**23**: 535-58.
- Svensson, C. K. (2003). "Do arylhydroxylamine metabolites mediate idiosyncratic reactions associated with sulfonamides?" Chem Res Toxicol**16**: 1035-43.
- Swinson, D. R., Zlosnick, J. & Jackson, L. (1981). "Double-blind trial of dapsone against placebo in the treatment of rheumatoid arthritis." Ann Rheum Dis**40**: 235-9.
- Tanvarasethee, B., Buranapraditkun, S. & Klaewsongkram, J. (2013). "The potential of using enzyme-linked immunospot to diagnose cephalosporin-induced maculopapular exanthems." Acta Derm Venereol**93**: 66-9.
- Tchou, I., Misery, L., Sabido, O., Dezutter-Dambuyant, C., Bourlet, T., Moja, P., Genin, C. (2001). "Functional HIV CXCR4 coreceptor on human epithelial Langerhans cells and infection by HIV strain X4." J Leukoc Biol, **70**(2): 313–321.
- Tew, K.D. et al., (2012). "The Role of Glutathione S-Transferase P in signalling pathways and S-glutathionylation in cancer." Free Radical Biology & Medicine**51**(2): 299–313.
- Tewary, P., Yang, D., De La Rosa, G., Li, Y., Finn, M. W., Krensky, A. M., Clayberger, C. & Oppenheim, J. J. (2010). "Granulysin activates antigen-presenting cells through TLR4 and acts as an immune alarmin." Blood**116**: 3465-74..
- Thong, B. Y. & Tan, T. C. (2011). "Epidemiology and risk factors for drug allergy." Br J Clin Pharmacol**71**: 684-700.

Bibliography

- Thuong-Nguyen, V.U., Kadunce, D.P., Hendrix, J.D., Gammon, W.R. and Zone, J.J., (1993). "Inhibition of Neutrophil Adherence to Antibody by Dapsone: A Possible Therapeutic Mechanism of Dapsone in the Treatment of IgA Dermatoses." Journal of investigative dermatology **100**(4): 349-355.
- Tian, W., Shen, J., Zhou, M., Yan, L. and Zhang, G., (2012). "Dapsone hypersensitivity syndrome among leprosy patients in China." Lepr Rev **83**(4): 370-377.
- Tingle, M.D., Mahmud, R., Maggs, J.L., Hawley, S., Coleman, M.D., Ward, S.A., & Park, B.K. (1988). "The effect of 2,2%-substitution on the metabolism and toxicity of dapsone in vitro and in vivo." Environmental Toxicology and Pharmacology **5**: 145–153.
- Tiwari, A. K., Mishra, A. K., Bajpai, A., Mishra, P., Sharma, R. K., Pandey, V. K. & Singh, V. K. (2006). "Synthesis and pharmacological study of novel pyrido-quinazolone analogues as anti-fungal, antibacterial, and anticancer agents." Bioorg Med Chem Lett **16**: 4581-5.
- Torosantucci, R., Brinks, V., Kijanka, G., Halim, L. A., Sauerborn, M., Schellekens, H., and Jiskoot, W.(2014). "Development of a transgenic mouse model to study the immunogenicity of recombinant human insulin." Journal of pharmaceutical sciences **103**(5): 1367-1374.
- Torres, M. J., Blanca, M., Fernandez, J., Romano, A., Weck, A., Aberer, W., Brockow, K., Pichler, W. J. & Demoly, P. (2003). "Diagnosis of immediate allergic reactions to beta-lactam antibiotics." Allergy **58**: 961-72.
- Torres, M. J., Gonzalez, F. J., Mayorga, C., Fernandez, M., Juarez, C., Romano, A. & Blanca, M. (1997). "IgG and IgE antibodies in subjects allergic to penicillins recognize different parts of the penicillin molecule." Int Arch Allergy Immunol **113**: 342-4.
- Tournade, H. et al., (1990). "D-penicillamine-induced autoimmunity in Brown-Norway rats. Similarities with HgCl₂-induced autoimmunity." Journal of immunology, **144**(8): 2985–91.
- Trepanier, L.A., (2004). "Idiosyncratic toxicity associated with potentiated sulfonamides in the dog." Journal of Veterinary Pharmacology and Therapeutics, **27**(3): 129–138.
- Trombetta, E. S., and Ira Mellman.(2005) "cell biology of antigen processing in vitro and in vivo." Annual Review of Immunology, **23**(1): 975-1028.
- Uetrecht J., (2005). "Current trends in drug-induced autoimmunity" Autoimmunity Reviews **4**(5): 309-14.
- Uetrecht, J. (2005). "Role of animal models in the study of drug-induced hypersensitivity reactions." AAPS J **7**: 914-21.
- Uetrecht, J. (2007). "Idiosyncratic drug reactions: current understanding." Annu Rev Pharmacol Toxicol **47**: 513-39.

Bibliography

- Uetrecht, J. (2008). "Idiosyncratic drug reactions: past, present, and future." Chem Res Toxicol **21**: 84-92.
- Uetrecht, J. and Naisbitt, D.J., (2013). "Idiosyncratic adverse drug reactions: current concepts." Pharmacological reviews **65**(2): 779-808.
- Uetrecht, J. P. (1999). "New concepts in immunology relevant to idiosyncratic drug reactions: the "danger hypothesis" and innate immune system." Chem Res Toxicol **12**: 387-95.
- Uetrecht, J., Zahid, N., Shear, N. H. & Biggar, W. D. (1988). "Metabolism of dapsone to a hydroxylamine by human neutrophils and mononuclear cells." J Pharmacol Exp Ther **245**: 274-9.
- Uetrecht, J.P. et al., (2010). "Nevirapine hypersensitivity." Handbook of Experimental Pharmacology **196**: 437–451.
- Uetrecht, J.P., (1992). The role of leukocyte-generated reactive metabolites in the pathogenesis of idiosyncratic drug reactions." Drug Metab Rev, **24**(3): s299–366.
- Umamaheswaran, G., Kumar, D.K. & Adithan, C., 2014. Distribution of genetic polymorphisms of genes encoding drug metabolizing enzymes & drug transporters - a review with Indian perspective. The Indian journal of medical research, **139**(1), pp.27–65.
- Vadher, A. and Lalljee, M., (1992). "Patient treatment compliance in leprosy; a critical review." International journal of leprosy and other mycobacterial diseases **60**(4): 587-607.
- Van Neerven, R. J., Wikborg, T., Lund, G., Jacobsen, B., Brinch-Nielsen, A., Arnved, J. & Ipsen, H. (1999). "Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4+ T cells by inhibiting serum-IgE-facilitated allergen presentation". J Immunol **163**: 2944-52.
- Vatsis, K., Weber, W., Bell, D., Dupret, J., Evans, D., Grant, D., Hein, D., Lin, H., Meyer, U., Relling, M., Sim, E., Suzuki, T. and Yamazoe, Y. (1995). Nomenclature for N-acetyltransferases. Pharmacogenetics, **5**(1), pp.1-17.
- Vieira, J.L., Riveira, J.G., Martins, A., Silva, J.P., & Salgado, C.G. (2010). "Methemoglobinemia and dapsone levels in patients with leprosy." Braz j infect Dis **14**:319–32
- Vinod, K. V., Arun, K., & Dutta, T. K. (2013). "Dapsone hypersensitivity syndrome: A rare life threatening complication of dapsone therapy." Journal of pharmacology & pharmacotherapeutics **4**(2): 158.
- Von Greyerz, S., Zanni, M.P., Frutig, K., Schnyder, B., Burkhart, C. and Pichler, W.J., (1999). "Interaction of sulfonamide derivatives with the TCR of sulfamethoxazole-specific human $\alpha\beta$ + T cell clones." The Journal of Immunology **162**(1): 595-602.

Bibliography

- Vree, T. B., & Hekster, Y. A. (1986). "Clinical pharmacokinetics of sulfonamides and their metabolites: an encyclopedia." Antibiotics and chemotherapy**37**: 1-214.
- Vyas, P.M. et al., (2006). "Enzyme-mediated protein haptation of dapsone and sulfamethoxazole in human keratinocytes: I. Expression and role of cytochromes P450." The Journal of pharmacology and experimental therapeutics**319**(1): 488–496.
- Vyas, P.M., Roychowdhury, S. and Svensson, C.K., (2006). "Role of human cyclooxygenase-2 in the bioactivation of dapsone and sulfamethoxazole". Drug metabolism and disposition**34**(1): 16-18.
- Walsh, S.A. and Creamer, D., (2011). "Drug reaction with eosinophilia and systemic symptoms (DRESS): a clinical update and review of current thinking." Clinical and experimental dermatology**36**(1): 6-11.
- Wang, N., Parimi, L., Liu, H. and Zhang, F., (2017). "A Review on Dapsone Hypersensitivity Syndrome Among Chinese Patients with an Emphasis on Preventing Adverse Drug Reactions with Genetic Testing." The American journal of tropical medicine and hygiene**96**(5): 1014-1018.
- Wang, R. and Green, D.R., (2012). "Metabolic checkpoints in activated T cells." Nature immunology**13**(10): 907-915.
- Warshakoon, H. J., Burns, M. R. & David, S. A. (2009). "Structure-activity relationships of antimicrobial and lipoteichoic acid-sequestering properties in polyamine sulfonamides." Antimicrob Agents Chemother**53**: 57-62.
- Watkins, S. and Pichler, W.J., (2013). "Sulfamethoxazole induces a switch mechanism in T cell receptors containing TCRV β 20-1, altering pHLA recognition." PloS one,**8**(10): 76211
- Watts, C. & Lanzavecchia, A. (1993). "Suppressive effect of antibody on processing of T cell epitopes." J Exp Med,**178**: 1459-63.
- Webster G. (2010). "Is topical dapsone safe in glucose-6-phosphate dehydrogenase-deficient and sulfonamide-allergic patients?" J Drugs Dermatol,**9**(5):532-6.
- Wester, K., Jonsson, A. K., Spigset, O., Druid, H. & Hagg, S. (2008). "Incidence of fatal adverse drug reactions: a population based study." Br J Clin Pharmacol,**6**., 573-9.
- White, T. J., Arakelian, A. & Rho, J. P. (1999). "Counting the costs of drug-related adverse events." Pharmacoeconomics **15**: 445-58.
- Whritenour, J., Cole, S., Zhu, X., Li, D. and Kawabata, T.T., (2014). "Development and partial validation of a mouse model for predicting drug hypersensitivity reactions." Journal of immunotoxicology, **11**(2): 141-147.

Bibliography

- Wozel, G. & Blasum, C. (2014). "Dapsone in dermatology and beyond." Arch Dermatol Res**306**: 103-24.
- Wright, S. W. & Hallstrom, K. N. (2006). "A convenient preparation of heteroaryl sulfonamides and sulfonyl fluorides from heteroaryl thiols." J Org Chem**71**: 1080-4.
- Wu, C., Bell, C.M. and Wodchis, W.P., (2012). "Incidence and economic burden of adverse drug reactions among elderly patients in Ontario emergency departments." Drug safety, **35**(9): 769-781.
- Wu, J. J., Huang, D. B., Pang, K. R., Hsu, S., & Tying, S. K. (2005). "Thalidomide: dermatological indications, mechanisms of action and side-effects." British Journal of dermatology**153**(2): 254-273.
- Wu, T. Y., Jen, M. H., Bottle, A., Molokhia, M., Aylin, P., Bell, D. & Majeed, A. (2010). "Ten-year trends in hospital admissions for adverse drug reactions in England 1999-2009." J R Soc Med**103**: 239-50.
- Wu, Y., Farrell, J., Pirmohamed, M., Park, B. K. & Naisbitt, D. J. (2007). "Generation and characterization of antigen-specific CD4+, CD8+, and CD4+CD8+ T-cell clones from patients with carbamazepine hypersensitivity." J Allergy Clin Immunol**119**: 973-81.
- Yawalkar, N. & Pichler, W. J. (2001). "Pathogenesis of drug-induced exanthema." Int Arch Allergy Immunol:**124**, 336-8.
- Yin, H., Anders, M.W., Korzekwa, K.R., Higgins, L., Thummel, K.E., Kharash, E.D., Jones, J.P., (1995). "Designing safer chemicals: Predicting the rates of metabolism of halogenated alkanes." Proc. Natl. Acad. Sci. U.S.A**92**: 11076–11080.
- Yip, V. L. M. (2016). "Carbamazepine hypersensitivity: linking metabolism to the immune response" (Doctoral dissertation, University of Liverpool).
- Yun, J., Adam, J., Yerly, D. & Pichler, W. J. (2012). "Human leukocyte antigens (HLA) associated drug hypersensitivity: consequences of drug binding to HLA." Allergy**67**: 1338-46.
- Yun, J., Cai, F., Lee, F.J. and Pichler, W.J., (2016). "T-cell-mediated drug hypersensitivity: immune mechanisms and their clinical relevance." Asia Pacific Allergy**6**(2): 77.
- Yun, J., Marcaida, M.J., Eriksson, K.K., Jamin, H., Fontana, S., Pichler, W.J. and Yerly, D., (2014). "Oxypurinol directly and immediately activates the drug-specific T cells via the preferential use of HLA-B* 58: 01" The Journal of Immunology**192**(7): 2984-2993.
- Yun, J., Mattsson, J., Schnyder, K., Fontana, S., Largiadèr, C.R., Pichler, W.J. and Yerly, D., (2013). "Allopurinol hypersensitivity is primarily mediated by dose-dependent oxypurinol-specific T cell response." Clinical & Experimental Allergy**43**(11): 1246-1255.

Bibliography

- Zanni, M.P., von Greyerz, S., Schnyder, B., Brander, K.A., Frutig, K., Hari, Y., Valitutti, S. and Pichler, W.J., (1998). "HLA-restricted, processing-and metabolism-independent pathway of drug recognition by human alpha beta T lymphocytes." Journal of Clinical Investigation**102**(8): 1591.
- Zawodniak, A., Lochmatter, P., Yerly, D., Kawabata, T., Lerch, M., Yawalkar, N. & Pichler, W. J. (2010). "In vitro detection of cytotoxic T and NK cells in peripheral blood of patients with various drug-induced skin diseases." Allergy**65**: 376-84.
- Zhang, F.R., Liu, H., Irwanto, A., Fu, X.A., Li, Y., Yu, G.Q., Yu, Y.X., Chen, M.F., Low, H.Q., Li, J.H. and Bao, F.F., (2013). "HLA-B* 13: 01 and the dapsone hypersensitivity syndrome." New England Journal of Medicine, **369**(17): 1620-1628.
- Zhu, Y.I. and Stiller, M.J., (2001). "Dapsone and sulfones in dermatology: overview and update." Journal of the American Academy of dermatology**45**(3): 420-434.
- Zuidema, J., Hilbers-Modderman, E. S. & Merkus, F. W. (1986). "Clinical pharmacokinetics of dapsone." Clin Pharmacokinet**11**: 299-315.